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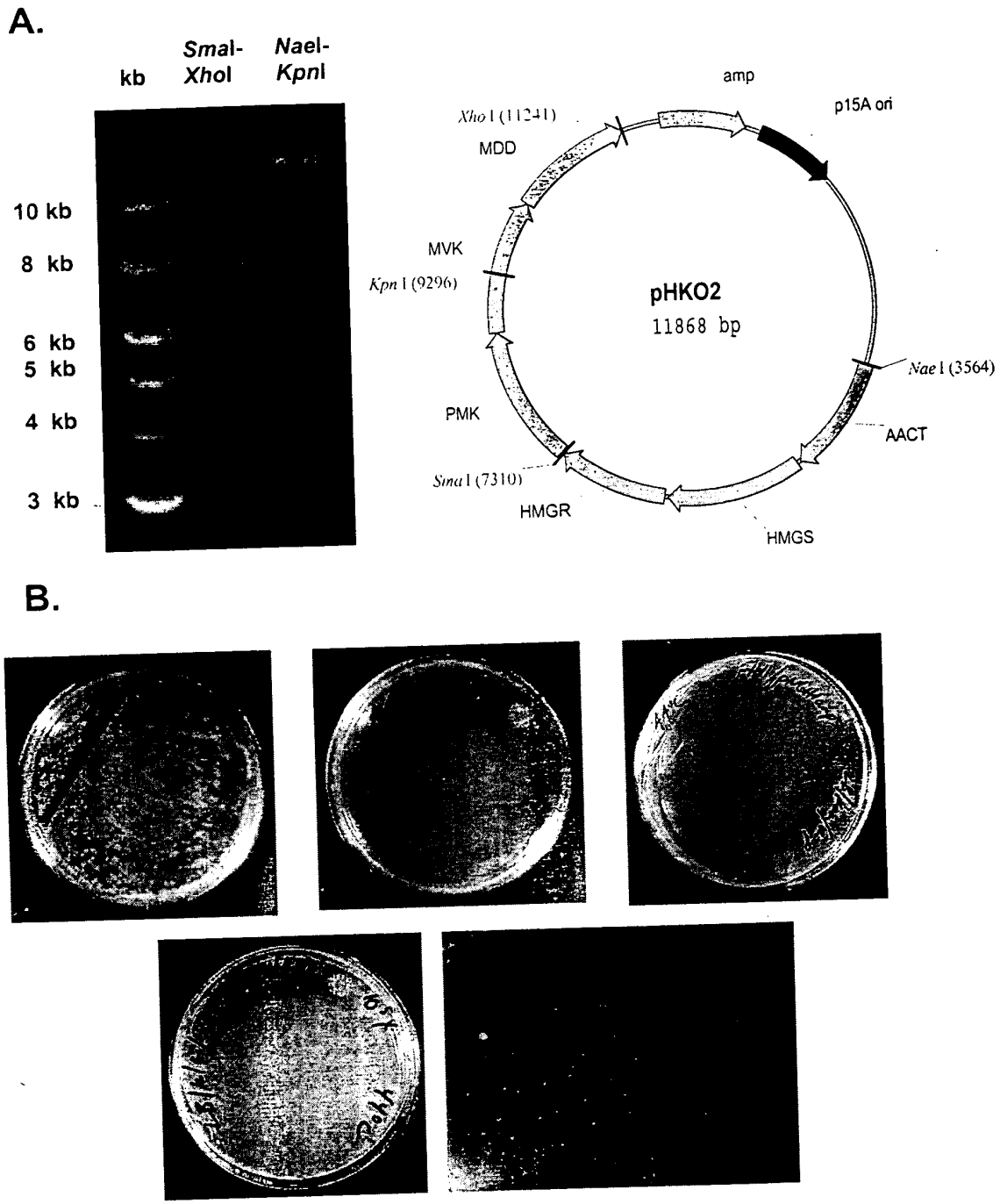

Fig. 2. A) Map and restriction analysis of vector pHKO2 containing orfs encoding acetoacetyl-CoA thiolase (AACT), HMG-CoA synthase (HMGS), HMG-CoA reductase (HMGR), phosphomevalonate kinase (PMK), mevalonate kinase, (MVK), and mevalonate diphosphate decarboxylase (MDD). **B)** FH11 cells containing lethal temperature sensitive *dxs*::kan^r (Hahn et al., 2001) incubated at 30 and 44 °C. FH11 cells containing pNGH1amp, parent vector of pHKO2, are unable to grow at the restrictive temperature. FH11/pHKO2 cells are able to grow at 44 °C demonstrating the ability of the mevalonate pathway orfs to synthesize IPP from acetyl-CoA in vivo and circumvent the *dxs* mutation in strain FH11.

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REVIEW ARTICLE

Transgenic Plastids in Basic Research and Plant Biotechnology

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Facile methods of genetic transformation are of outstanding importance for both basic and applied research. For many years, transgenic technologies for plants were restricted to manipulations of the nuclear genome. More recently, a second genome of the plant cell has become amenable to genetic engineering: the prokaryotically organized circular genome of the chloroplast. The possibility to directly manipulate chloroplast genome-encoded information has paved the way to detailed *in vivo* studies of virtually all aspects of plastid gene expression. Moreover, plastid transformation technologies have been intensely used in functional genomics by performing gene knockouts and site-directed mutageneses of plastid genes. These studies have contributed greatly to our understanding of the physiology and biochemistry of biogenergetic processes inside the plastid compartment. Plastid transformation technologies have also stirred considerable excitement among plant biotechnologists, since transgene expression from the plastid genome offers a number of most attractive advantages, including high-level foreign protein expression and transgene containment due to lack of pollen transmission. This review describes the generation of plants with transgenic plastids, summarizes our current understanding of the transformation process and highlights selected applications of transplastomic technologies in basic and applied research.

Keywords: chloroplast; plastid transformation; reverse genetics; functional genomics; plant biotechnology

Introduction

The genetic information of plants is distributed among three cellular compartments: the nucleus, the mitochondria and the plastids. The latter two are derived from formerly free-living eubacteria: mitochondria from α-proteobacteria and plastids from cyanobacteria. The prokaryotic progenitors of the present-day cell organelles were engulfed by a pre-eukaryotic cell in a endosymbiosis-like process. During the gradual integration of the acquired endosymbionts into the host cell's metabolism, the organellar genomes underwent a dramatic size reduction due to both massive gene loss and gene transfer to the nuclear genome.[1] Consequently, present-day organellar genomes are rather small and contain comparably little information. The plastid genome is a circular molecule of double-stranded DNA. In a typical higher plant, it is 120–160 kb in size and contains approximately 130 genes. Identical copies of the plastid genome are present in all plastid differentiation types: proplastids (predominantly present in meristematic tissues), green chloroplasts (present in photosynthetically active tissues), carotenoid-accumulating red or yellow chromoplasts (present in some flowers and fruits) as well as several other plastid types specialized in storage of starch, lipids or proteins.

In spite of the small size of plastid genomes as compared with higher plant nuclear genomes, chloroplast DNA typically makes up as much as 10–20% of the total cellular DNA content.[2] This is because a diploid plant cell harbors only two

Abbreviations used: ptDNA, plastid DNA; PEG, polyethylene glycol; GUS, β-glucuronidase; GFP, green fluorescent protein; UTR, untranslated region; *ycf*, hypothetical chloroplast reading frame.

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copies of its nuclear genome but thousands of copies of its plastid genome (see Figure 2). A single leaf cell may contain dozens or even hundreds of chloroplasts.[2] The chloroplast DNA is organized in nucleoids as typical of a prokaryotic system and several such nucleoids are present in each chloroplast. Each nucleoid again harbors several copies of the plastid genome (see Figure 2). This can add up to extraordinarily high ploidy levels of the plastid genome: up to 10,000 (identical) copies of the plastid DNA (ptDNA) can be found in a single pea leaf cell and even up to 50,000 copies in a wheat cell.[2]

Plastids (as well as mitochondria) have retained numerous eubacterial features, including, for example, gene organization in operons and principally prokaryotic mechanisms of gene expression. Over the past decades, the plastid genome, its structure, expression and evolution was intensely studied using molecular methods. This research has generated a wealth of new information not only about the function of the plastid genetic system but also about the highly sophisticated regulatory mechanisms governing the co-operation of plant cell organelles with their nucleo-cytoplasmic compartment (for reviews see, e.g. Goldschmidt-Clemont,[3] Leon *et al.*[4] and Coleman and Nerozzi[5]). More recently, chloroplast research has benefited enormously from the introduction of transgenic technologies facilitated by the development of reliable methods for plastid genome transformation.[6–8] This methodological breakthrough has made feasible the targeted manipulation of the endogenous genetic information of plastids and, in addition, has opened up the exciting possibility to introduce novel information and express it from engineered chloroplast genomes.

Plastid transformation systems

For many years, the genetic transformation of organellar genomes seemed impossible to achieve, since (a) the double membrane of chloroplasts and mitochondria posed a threatening physical barrier to the delivery of transforming DNA into organelle compartments, and (b) no viruses or bacteria were known that would infect chloroplasts or mitochondria and thus could be used as vehicles for gene transfer. This rather pessimistic view changed suddenly when a new "violent" method was introduced into biological research: the shooting with particle-accelerating devices nowadays commonly called "particle guns" or "gene guns".[9,10] Together with the development of efficient protocols for coating inert metal powder (gold or tungsten) with nucleic acids, this biolistic (biological + ballistic) technique has provided the attractive opportunity to shoot foreign DNA into living cells.[11] Encouraged by promising success with nuclear transformation in plants[12] and mitochondrial transformation in yeast,[13] chloroplasts were the next targets of the cannoneers among plant researchers. In 1988, successful chloroplast transformation was reported by the Boynton and Gillham laboratories for *Chlamydomonas reinhardtii*, a unicellular green alga with a single large chloroplast occupying approximately 60% of the cell volume.[6] Employing photosynthetically incompetent mutants carrying defective alleles of the chloroplast *atpB* gene (and thus lacking chloroplast ATP synthase activity), the wild-type *atpB* gene was used in this study to complement the mutant phenotype under selection for restored photoautotrophic growth. Stable chloroplast transformants were obtained in which the mutant *atpB* allele had been replaced by the wild-type gene as present in the transformation vector *via* homologous recombination.

A seminal contribution to the further improvement of chloroplast transformation technologies was the development of the first chloroplast-specific antibiotic resistance marker, an originally bacterial aminoglycoside 3"-adenylyltransferase gene (*aadA*) conferring resistance to a number of antibiotics of the aminoglycoside type, including spectinomycin and streptomycin.[14] The antibiotic routinely used for chloroplast transformation is spectinomycin because of its high specificity as a prokaryotic translational inhibitor and its low side-effects on plant cells. The AadA protein catalyzes the covalent transfer of an AMP residue from ATP to spectinomycin, thereby converting the antibiotic into an inactive form (adenylylspectinomycin) that no longer inhibits protein biosynthesis on prokaryotic 70 S ribosomes as present in the chloroplast. In order to convert the *aadA* gene from *Escherichia coli* into a chloroplast-specific selectable marker, its coding region was fused to chloroplast expression signals:[14] a 5' DNA segment providing promoter, 5' untranslated region (UTR) and Shine-Dalgarno sequence as well as a 3' chloroplast DNA segment providing a stable 3' UTR which is required to confer transcript stability *in planta*.

In 1989, Pal Maliga and co-workers were the first gunners to succeed with chloroplast transformation in a higher plant.[7] Using a chloroplast 16 S ribosomal RNA gene engineered by introducing point mutations that confer resistance to spectinomycin and streptomycin, they demonstrated stable transformation of tobacco (*Nicotiana tabacum*) plastids by biolistic bombardment of sterile leaves followed by selection for spectinomycin-resistant cell lines (Figure 1). In the chloroplast genomes of the transformed plants (also referred to as "transplastomic" plants), the engineered 16 S rRNA allele as present in the transformation vector had replaced the wild-type allele by homologous recombination.[7] Reciprocal crosses of transplastomic and wild-type plants demonstrated that the introduced antibiotic resistances were uniparentally, maternally inherited as expected for an extranuclear trait.

The initially used antibiotic-resistant 16 S rRNA allele was not an efficient selectable marker and produced on average only one or two tobacco chloroplast transformants per 100 bombarded leaf samples (equaling approximately 400 selection

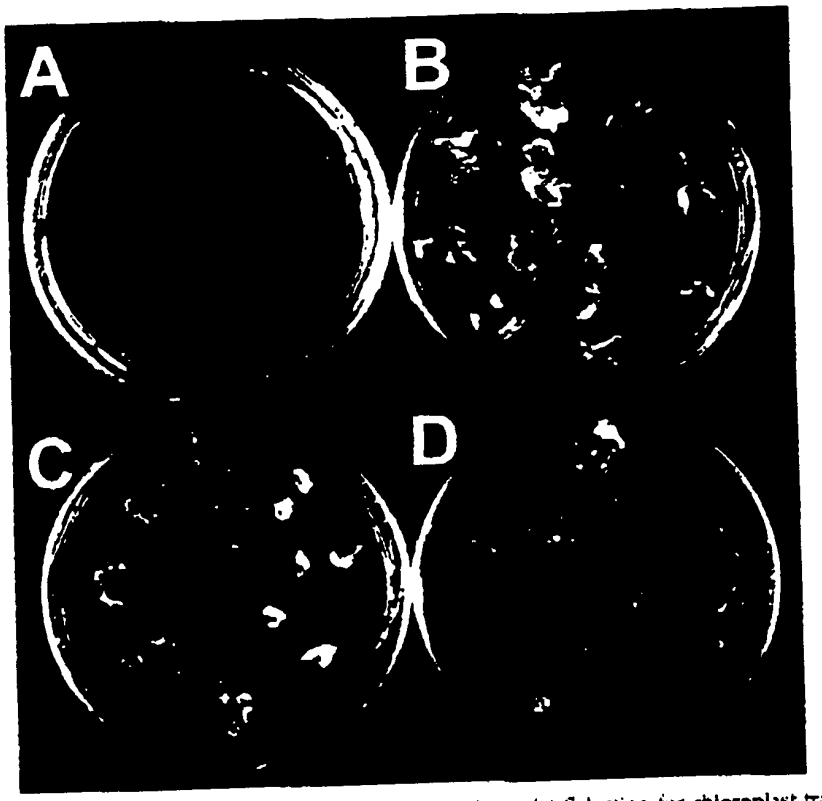

Figure 1. Generation of tobacco plants with transgenic chloroplasts. (a) Selection for chloroplast transformants. Following biolistic bombardment, sterile tobacco leaves are cut into small pieces and exposed to spectinomycin-containing plant regeneration medium. (b) After incubation for four weeks, the leaf pieces are completely bleached out due to effective inhibition of plastid protein biosynthesis by spectinomycin (an aminoglycoside antibiotic specifically blocking translation on prokaryotic-type 70 S ribosomes). A successfully transformed chloroplast expresses the spectinomycin-resistance gene, *aadA*, thus allowing for continued cell and organelle divisions in the presence of the antibiotic. Resistant cell populations initially grow as undifferentiated green callus tissue before the phytohormones present in the synthetic medium eventually induce shoot formation. (c) Elimination of spontaneous spectinomycin-resistant mutants by double selection. Spontaneous spectinomycin resistance occurs through acquisition of specific point mutations in the chloroplast 16 S rRNA gene.[16] Such point mutations act in a strictly antibiotic-specific manner: spontaneous spectinomycin-resistant cells are streptomycin sensitive and *vice versa*. In contrast, the *aadA* transgene confers broad-spectrum resistance to a number of antibiotics of the aminoglycoside type. Consequently, when exposed to double selection on plant regeneration medium containing both spectinomycin and streptomycin, leaf pieces from spontaneous spectinomycin-resistant lines bleach out (right three lines), whereas leaf pieces from true chloroplast transformants remain green, form calli and ultimately regenerate new plantlets. (d) Selection of homoplasmic transplastomic lines. As primary chloroplast transformants are heteroplasmic (see Figure 2), they must be subjected to several additional rounds of regeneration on selective medium in order to eliminate residual wild-type genomes and establish cell lines carrying a uniform population of transformed plastid genomes. To this end, leaf explants are taken in each round and re-exposed to spectinomycin-containing plant regeneration medium.

plates; Figure 1(a)).[7,15,16] This relatively low transformation frequency is most probably due to the recessive mode of action of the rRNA marker during the selection phase: It confers antibiotic resistance only to those few chloroplast ribosomes that have received their 16 S rRNA molecule from the very few initially present transformed ptDNA copies (Figure 2). By contrast, antibiotic-inactivating marker genes provide dominant drug resistance to the recipient chloroplast and, in theory, a single transformed genome copy is sufficient to detoxify the entire organelle. Two such dominant selectable marker genes for tobacco chloroplast transformation have been constructed to date (Table 1): (a) a chimeric spectinomycin resistance gene *aadA*[8] as described above for *Chlamydomonas* chloroplast transformation[14] but containing tobacco chloroplast-specific expression signals; and (b) a similarly designed chimeric *nptII* gene encoding a neomycin phosphotransferase and conferring resistance to kanamycin.[17,18] While the *aadA* gene is a highly efficient and specific selectable marker, the *nptII* appears to be less efficient and, moreover, produces a significant background of nuclear transformants.[17]

Table 1. Foreign genes successfully expressed to date from higher plant plastid genomes

Gene(s)	Gene product and gene source	Function	References
aadA	Aminoglycoside 3′-adenylyltransferase from *E. coli*	Positive selectable marker (spectinomycin and streptomycin resistance)	8, 107
nptII	Neomycin phosphotransferase from Tn5	Positive selectable marker (kanamycin resistance)	17, 18
uidA	β-Glucuronidase (GUS) from *E. coli*	Reporter of gene expression	16,108
GFP	Green fluorescent protein (GFP) from *Aequorea victoria*	(Vital) reporter of gene expression	99, 100
cry1A	Crystal toxin from *Bacillus thuringiensis*	Insecticidal protein (protoxin)	82
cry2A	Crystal toxin from *Bacillus thuringiensis*	Insecticidal protein (protoxin)	101
cry2A2 operon	Crystal toxin, ORF1 and ORF2 (putative chaperonin) proteins from *Bacillus thuringiensis*	Insecticidal protein (protoxin)	84
codA	Cytosine deaminase from *E. coli*	Negative selectable marker (5-fluorocytosine sensitivity)	63
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase from *Petunia hybrida* or from eubacterial species	Herbicide tolerance (glyphosate)	96, 83
bar	Phosphinothricin acetyltransferase from *Streptomyces hygroscopicus*	Herbicide resistance (glufosinate)	91
hSI	Human somatotropin	Therapeutic protein (human growth hormone)	103

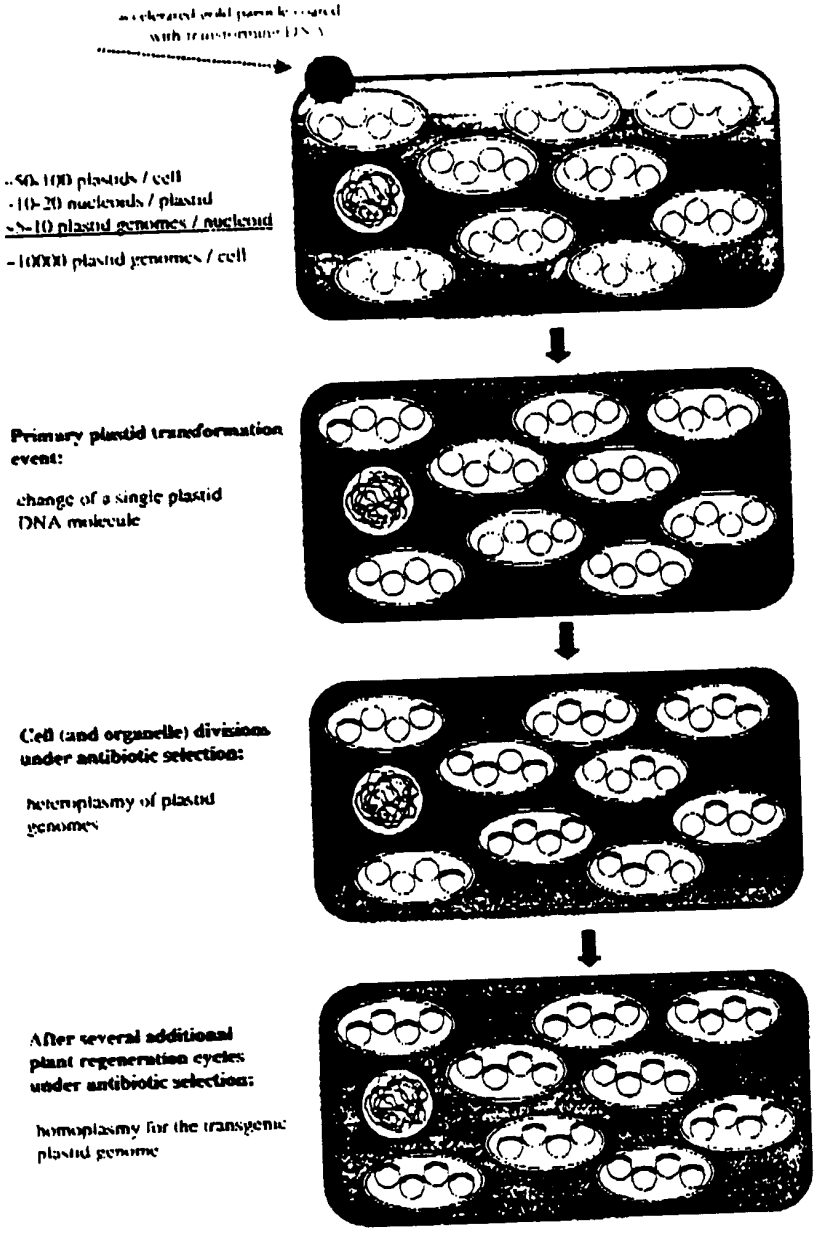

Figure 2. Sorting of plastid genomes and isolation of homoplasmic transplastomic cell lines. The initial chloroplast transformation event involves the change of only a single (or at most a few) out of several thousand plastid genome copies in a leaf cell. During subsequent cell and organelle divisions, the presence of high concentrations of the selecting antibiotic favors multiplication of chloroplasts containing transformed genomes, whereas chloroplasts harboring only wild-type genomes may be eliminated effectively. However, individual chloroplasts may still contain a mixed population of wild-type and transformed plastid genome molecules (intraorganellar heteroplasmy). In additional rounds of plant regeneration on selective medium, gradual sorting out of residual wild-type genomes is achieved, eventually leading to cells with a homogeneously transformed population of plastid genomes commonly referred to as "homoplasmic" or "homoplastidic". See the text for details.

In summary, three types of selectable markers are available for chloroplast transformation experiments: (i) dominant antibiotic-resistance genes (available for *Chlamydomonas* and tobacco[8,14,17,19]); (ii) recessive antibiotic-resistance markers encoding antibiotic-insensitive alleles of ribosomal RNA genes (available for *Chlamydomonas* and tobacco[7,20]); and (iii) recessive markers restoring photoautotrophic growth by complementing non-photosynthetic mutants (currently only available

for *Chlamydomonas*[6]). As outlined above, dominant spectinomycin resistance provided by chimeric *aadA* genes is by far the most effective selection system for chloroplast transformants available to date.

Although direct gene transfer using the biolistic method is undoubtedly the currently most widespread technology for plastid transformation, stable introduction of cloned DNA into chloroplast genomes also has been conclusively demonstrated using two alternative protocols. For *Chlamydomonas*, agitating a suspension of glass beads and cell wall-deficient algal cells in the presence of plasmid DNA produced transplastomic cells, albeit at significantly lower rate than the biolistic protocol.[21] In tobacco, chloroplast transformation can alternatively be accomplished by chemical treatment of protoplasts with polyethylene glycol (PEG) in the presence of vector DNA.[22-24] PEG had long been known to promote uptake of naked DNA by protoplasts and was routinely used to deliver biologically active DNA into the plant nuclear genome.[25] Its suitability for the transformation of chloroplasts was somewhat surprising, since it is generally believed that direct exposure of membranes to high PEG concentrations is required to facilitate the passage of DNA molecules. Although in electron microscopy, mesophyll chloroplasts are often seen tightly appressed to the plasma membrane, it is by no means clear how such a membrane permeabilization by PEG could work for the double membrane of the chloroplast when intact protoplasts are treated with PEG. Nonetheless, PEG-mediated plastid transformation in tobacco protoplasts, followed by regeneration of genetically stable transplastomic plants appears to be a reliable and reproducible technique and has been used successfully by several laboratories.[26-28]

In addition to technologies for stable genetic transformation of plastids, several methods for transient gene expression have been described, including *in organello* systems introducing DNA into isolated plastids[29,30] and *in vivo* methods employing particle bombardment[31-33] or microinjection techniques.[34]

The molecular biology of the transformation process

Stable plastid transformation in both *Chlamydomonas* and tobacco appears to be strictly dependent on integration of the transforming DNA into the plastid genome by homologous recombination. Fortunately, plastids have inherited from their cyanobacterial ancestors an efficient RecA-type system of homologous recombination.[35] Any plastid genome manipulation therefore requires that the sequence to be introduced into the plastid genome is flanked on both sides by regions of homology with the chloroplast genome.[15,27] Although the minimum sequence requirements for efficient homologous recombination to occur are currently not very well defined, it is generally assumed that upwards of approximately 400 bp flanking region on each side, chloroplast transformants are obtained at reasonable frequency. Longer flanks appear to be beneficial, but no careful correlation between size of the homologous regions and transformation frequency has been established to date.

It seems reasonable to assume that the primary plastid transformation event involves the change of only a single or at most a few plastid genome copies (within a single chloroplast) out of the ~10,000 ptDNA copies present in a leaf mesophyll cell (Figure 2). Consequently, primary transplastomic cell lines contain a mixed population of wild-type and transformed plastid genomes (Figure 2). Such cells, tissues or plants are also referred to as heteroplasmic (or, more specifically, "heteroplastomic"). It has been known for almost a century[36,37] that heteroplasmic situations are genetically unstable and, more or less frequently, resolve spontaneously into either of the two types of genome homogeneity ("homoplasmy"). This sorting-out of extranuclear genetic material is due to random genome segregation upon organelle division as well as random organelle segregation upon cell division.

From this, it appears clear that genetic stability of transplastomic cell lines and plants requires homoplasmy. Homoplasmy can be achieved by allowing for a sufficient number of cell divisions under high selective pressure as exerted by high concentrations of the selecting antibiotic spectinomycin. For *Chlamydomonas*, this is simply done by re-streaking the growing colonies on fresh culture medium containing spectinomycin. For tobacco, plants with a uniform population of transformed genomes are obtained by passing the primary chloroplast transformant through additional cycles of plant regeneration under antibiotic selection: tissue samples are excised from regenerating shoots and re-exposed to regeneration medium with spectinomycin (Figure 1(d)). Typically, homoplasmic (or "homoplastomic") shoots are obtained after two to four such cycles of regeneration under selection. Highly sensitive assays have been developed for confirming homoplasmy and reliably proving the absence of any residual wild-type genome copies, including large-scale seed assays[6] and PCR-based tests strongly favoring amplification of wild-type genomes.[38]

In general, two levels of heteroplasmy must be distinguished: (a) interplastidic heteroplasmy, i.e. the presence of chloroplasts with wild-type genomes and those with mutant genomes within one and the same cell; and (b) intraplastidic heteroplasmy, i.e. the simultaneous presence of wild-type and mutant plastid genomes within one and the same chloroplast. Most likely, plastid transformation and gradual sorting out of wild-type genomes involves both types of heteroplasmy (Figure 2). Interplastidic heteroplasmy is likely to disappear rather rapidly, since chloroplasts exclusively harboring wild-type genomes are sensitive to the selecting antibiotic and hence will not multi-

ply as effectively as transformed chloroplasts. In contrast, intraplastidic heteroplasmy is probably more difficult to eliminate since the antibiotic-resistance gene acts as a dominant selectable marker in the sense that one or few copies of the resistance gene are sufficient to confer resistance to the entire organelle. Consequently, there is probably no significant selective advantage of becoming homoplasmic. Why then is it feasible to isolate homoplasmic chloroplast transformants after two to four rounds of plant regeneration on spectinomycin-containing medium? Assuming that, from a certain copy number of transplastomes onwards, antibiotic selection becomes neutral, sorting of genome types will be random upon plastid division. Random distribution of plastid genomes during the organelle division process will give rise occasionally to homoplasmic wild-type chloroplasts or homoplasmic transgenic chloroplasts. Whereas the homoplasmic transgenic chloroplasts will be (at least) as competitive as the heteroplasmic ones, the homoplasmic wild-type chloroplasts will be antibiotic-sensitive and thus may not multiply efficiently during subsequent cell divisions. This model might explain how, over time, wild-type genomes are gradually diluted out and eventually disappear.

There are a few examples of tobacco or *Chlamydomonas* chloroplast transformation experiments resulting in the formation of episomal, plasmid-like elements.[39-42] However, in all these cases, stable integration of the transforming DNA into the plastid genome by homologous recombination also occurred and hence it is not clear whether the episomal elements really replicate autonomously inside the chloroplast or alternatively reflect mini-circle-like recombination products continuously originating from the transformed plastid genome. Interestingly, biolistic transformation experiments in the green alga *Euglena gracilis* have provided evidence for successful chloroplast transformation in the absence of any integration of the transforming DNA into the chloroplast genome.[43] Instead, under selective conditions, transforming DNA was maintained as an episomal element at low copy number (approximately one copy per chloroplast). It is currently unclear why *Euglena gracilis* chloroplasts do not integrate transforming DNA into their genome by homologous recombination.

In vivo systems for studying plastid gene expression

The possibility to manipulate plastid DNA sequences *in vitro* and re-introduce the altered sequences into the chloroplast genome has also paved the way to study practically all mechanisms of plastid gene expression in *in vitro* systems. These studies have greatly contributed to our understanding of the rules governing plastid gene expression.[3,44-46]

The development of chimeric reporter genes for plastid gene expression has provided valuable tools for systematic studies of the *cis*-acting elements involved in transcription, RNA metabolism and translation.[40,47,28] The *uidA* gene encoding β-glucuronidase (GUS) has been used in most of these studies, but recent work has shown that the *gfp* gene (encoding the green fluorescent protein, GFP) also functions well in plastids (Table 1). When the coding region of the reporter is fused to plastid gene-specific expression signals, reporter gene expression reliably follows the pattern of the endogenous plastid gene,[46] indicating that the major *cis*-acting sequence elements determining RNA stability and controlling translational efficiency reside within the 5′ and 3′ UTRs of plastid messenger RNAs.

Transplastomic *in vivo* systems are particularly valuable for the study of all those steps in plastid gene expression for which no faithful or efficient *in vitro* systems are available (or have not been available until very recently), such as group II intron splicing and RNA editing. Tobacco plastid transformation, for example, has been extensively used to study RNA editing, a curious RNA processing step in higher plant cell organelles. RNA editing in plastids of vascular plants is a post-transcriptional process that changes individual cytidine residues into uridine.[47] Editing events usually result in changes of the coding properties of the affected mRNAs which has the dramatic consequence that amino acid sequences cannot be reliably predicted from DNA sequence analyses. As a faithful *in vitro* system for plastid RNA editing was lacking until very recently,[3] chloroplast transformation experiments have been the method of choice to address functional, mechanistic and evolutionary aspects of RNA editing.[48] Systematic deletional and point mutageneses have allowed to dissect the *cis*-acting elements involved in editing site recognition and to define minimum substrates for plastid editing reactions.[38,54-56] In addition, *in vitro* studies in transgenic chloroplasts have begun to shed some light on the molecular mechanism of the editing reaction[57] and on the evolution of editing sites and their *trans*-acting recognition factors.[58,59]

Transplastomic studies also have been instrumental in advancing our knowledge about mRNA synthesis and transcriptional regulation in plastids. The transcriptional apparatus of plastids comprises a plastid-encoded *E. coli*-like RNA polymerase and a recently identified second, nuclear-encoded transcription system utilizing a bacteriophage-type RNA polymerase.[60] Comparative studies of *in vitro* capped chloroplast transcripts from wild-type plants and transplastomic plants lacking the plastid-encoded RNA polymerase[61] have assigned transcription initiation sites to the two transcription systems and, in addition, have suggested consensus sequences for promoters recognized by the phage-type enzyme.[62] These studies have provided novel insights into the regulation of plastid gene expression in response to developmental and environmental cues.

Transplastomic approaches can also contribute to the identification of *trans*-acting factors involved in plastid gene expression. In this respect, negative selectable-marker genes are particularly useful since they allow to devise genetic screens for nuclear genes regulating plastid gene expression. A negative selectable marker for plastids has been developed from the *E. coli* cytosine deaminase gene (*codA*; Table 1) whose expression is lethal to the cell in the presence of exogenously applied 5-fluorocytosine.[63] Introduction into the ptDNA of the *codA* coding region fused to chloroplast gene-specific expression signals (promoter, 5' and 3' UTRs) combined with mutagenesis of the nuclear genome would be a highly efficient approach to isolate both general and gene-specific *trans*-acting factors involved in the expression of plastid genes at the transcriptional and post-transcriptional levels.

Chloroplast functional genomics by reverse genetics

Complete plastid genomes have been sequenced from a number of vascular plant and algal species†. The picture that has emerged from these extensive structural genomics studies is that the plastid genomes of green algae and higher plants are remarkably conserved in their coding capacity and genome organization. The majority of plastid-encoded genes can be grouped into two basic classes: genetic system genes (e.g. rRNA, tRNA and ribosomal protein genes) and photosynthesis-related genes.[64] In addition to the many functionally assigned genes, plastid genomes harbor a number of open reading frames of unknown function.[65] Those open reading frames that display a significant degree of interspecific conservation are generally considered to be genuine genes and are commonly referred to as *ycfs* (e.g. *ycf3*=hypothetical chloroplast reading frame number 3).

The availability of transgenic technologies for chloroplasts has facilitated the functional characterization of plastid genome-encoded genes and open reading frames using reverse genetics approaches. In contrast to forward genetics, where the (mutated) gene causing an interesting phenotype is aimed to be identified, reverse genetics starts from a known DNA sequence containing an open reading frame of unknown or uncertain function(s) and aims at its mutational inactivation *in vivo*. Lack of the gene product encoded by the reading frame of interest is hoped to produce an analyzable phenotype whose careful characterization is expected to reveal the function of the gene in the wild-type.

Owing to the efficient homologous recombination system in chloroplasts, reverse genetics by targeted knockout analysis or site-directed mutagenesis has become a powerful tool for plastid functional genomics.[66] As described above, chloroplast transformation technologies are nowadays routinely available for two model systems, the unicellular green alga *Chlamydomonas reinhardtii* and the higher plant tobacco. Both of these model plants have been used for systematic functional genomics in chloroplasts using reverse genetics strategies.[67-73]

A reverse genetics study in chloroplasts starts out with the construction of a mutant allele for the reading frame of interest by using standard *in vitro* techniques for insertional, deletional or site-directed mutagenesis. Linked to a selectable marker, the constructs are then introduced into the plastid genome by chloroplast transformation where the mutant allele replaces the endogenous wild-type allele by homologous recombination. Obviously, homoplasmy of the generated transplastomic lines is an absolute requirment for obtaining stable and clearly interpretable phenotypes.

Construction of a null allele by deletional or insertional mutagenesis is the most appropriate strategy in those cases where the function of an open reading frame (*ycf*) is entirely unknown. Such gene knockouts performed in *Chlamydomonas* and tobacco chloroplasts have led to the discovery of several new gene functions in the plastid genome including a number of small subunits of the large pigment-protein complexes involved in the light reactions of photosynthesis.[67,68,72,73] Interestingly, chloroplast gene disruptions also have identified novel proteins that are required for the stable accumulation of multiprotein complexes in the thylakoid membrane without being integral components of these complexes. Instead, these proteins may serve as important auxiliary factors in the assembly process of membrane protein complexes. Knockout analysis in chloroplasts have established, for example, that the proteins encoded by the conserved plastid reading frames *ycf3* and *ycf4* are essential factors for the assembly of stable photosystem I complexes in the thylakoid membrane.[70,71]

Homoplasmic transplastomic cells can be obtained for all knockouts of photosynthesis-related reading frames. This is because photosynthesis is not required under heterotrophic *in vitro* culture conditions: non-photosynthetic *Chlamydomonas* cells can be grown heterotrophically on acetate-containing medium and non-photosynthetic tobacco cells grow on sucrose-containing tissue culture media. However, for several plastid genome-encoded reading frames, the generation of homoplasmic knockout cells has turned out to be impossible indicating that these genes encode essential functions for cellular survival. Under selective conditions, these transplastomic lines remain heteroplasmic with wild-type and transformed genomes co-existing in a relatively constant ratio.[70,74] This stable heteroplasmy suggests a balanced selection in which the presence of both genome types is required for cell survival: whereas

† Available online (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/plastids_tax.html)

the transformed genome is essential to express the antibiotic resistance, the wild-type genome is required to provide the gene product of the essential gene (that was knocked-out in the transformed genome). Release of the selective pressure for maintenance of the transformed genome (by cultivation on antibiotic-free medium) allows for random sorting-out of plastid genomes and eventually results in the appearance of homoplasmic wild-type cells.[76]

In addition to knockout experiments with chloroplast open reading frames, reverse genetics analyses are also of great value for the detailed functional characterization of known plastid-encoded proteins by conducting site-directed mutageneses of the respective genes. In this way, crucial protein-protein interaction sites were mapped and important co-factor binding residues in electron-transferring proteins were identified.[77] These studies have contributed greatly to our understanding of the structure of membrane protein complexes and have significantly advanced our knowledge about the bioenergetic pathways in the chloroplast (reviewed by Hippler *et al.*[78]).

Transgenic chloroplasts in biotechnology

Plastid transformation technologies have attracted biotechnologists, since accommodation of transgenes in the plastid genome instead of the nuclear genome bears a number of inherent advantages for plant genetic engineering.[79–81]

Owing to the polyploidy of the plastid genetic system with thousands of genome copies per cell, extraordinarily high levels of foreign protein accumulation can be achieved in chloroplasts.[82–84] Transgenic plastids are thus ideal expression factories for high-yield protein production. Most remarkably, expression levels of up to more than 40 % of the total soluble cellular protein have been obtained[84] which is ten to 100 times higher than upon nuclear transgene expression in plants. As plastids have in their stroma a limited set of (prokaryotic) protein degradation pathways[85], it seems conceivable that at least some foreign proteins are not only produced to higher levels but are also more stable inside the chloroplast than in the nucleo-cytoplasmic compartment. However, to what extent protein stability contributes to the enormously high foreign protein accumulation levels in transgenic chloroplasts is currently unknown.

Another advantage of transplastomic technologies is that transgene expression is much more stable and uniform among transgenic lines. Nuclear transformation in plants occurs by more or less random integration of transgenes into unpredictable genomic locations through non-homologous recombination. This results in transgenic lines with widely varying expression levels (position effects) and usually requires screening of large numbers of transgenic plants to identify a line displaying reasonably high transgene expression. Additionally, nuclear transformation experiments in plants frequently suffer from epigenetic gene-inactivation mechanisms commonly referred to as gene silencing.[86] By occurring somatically, epigenetic gene inactivation may even cause variability in transgene expression levels within one plant.[87] By contrast, in plastid genomes, transgene integration always occurs by homologous recombination and is neither affected by position effects nor by epigenetic gene-silencing mechanisms. Thus, all transplastomic lines obtained from transformation experiments with a given vector are usually genetically and phenotypically identical and, in theory, the production of a single transplastomic plant per construct is sufficient. This at least partially compensates for the otherwise more laborious and technically demanding use of transplastomic approaches.

Plastid genome engineering also offers unique advantages for the simultaneous expression of multiple transgenes ("transgene stacking"). Transgene stacking is technically difficult in eukaryotic genomes, since multiple transgenes cannot be expressed by co-transcription. This is due to the mechanisms of translation initiation in eukaryotic cells[88] which normally permit only translation of the first cistron in a polycistronic messenger RNA. By contrast, the principally prokaryotic organization of plastid genomes allows expression of multiple transgenes from operons, since downstream cistrons of a polycistronic messenger RNA are faithfully translated.[84,89] Because related biosynthetic genes in bacteria are often organized into operons, this opens up the attractive possibility of introducing novel biosynthetic pathways into plastids by expressing entire bacterial operons. As an alternative to transgene stacking by expression as operons, co-transformation can also be used to insert multiple unlinked transgenes into the plastid genome. Moreover, techniques have been developed to recycle the antibiotic-resistance gene after successful plastid transformation. Such strategies for selectable marker removal from transplastomic cells involve either co-transformation or homologous recombination in direct repeats flanking the marker gene.[90,91]

Transplastomic technologies are also advantageous for ecological reasons. In the vast majority of angiosperm plant species, chloroplasts are passed uniparentally, maternally to the next generation (Figure 3).[92–94] This is due to either exclusion of plastids by unequal cell divisions upon pollen grain mitoses or degradation of plastids and plastid DNA during male gametophyte development.[95] Consequently, the sperm cell fertilizing the egg is free of plastids and plastid DNA, hence the zygote receives its plastids exclusively from the egg cell without any contribution from the pollen. This lack of pollen transmission of chloroplast genes and transgenes (Figure 3)[95,96] addresses two major public concerns about transgenic plants: (a) the probability of uncontrolled spreading of transgenes *via* pollen from fields with transgenic crops to fields

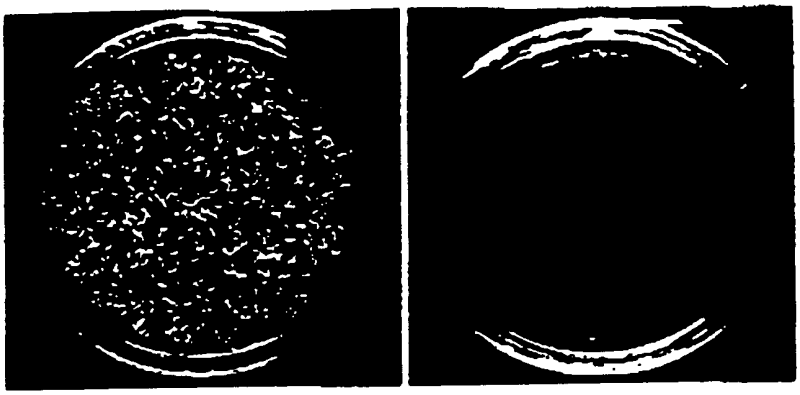

Figure 3. Lack of pollen transmission of chloroplast transgenes due to uniparental, maternal plastid inheritance. Seeds from reciprocal crosses of a transplastomic tobacco plant with a wild-type plant were germinated on spectinomycin-containing synthetic medium. F1 progeny obtained by pollination of wild-type flowers with pollen from a transplastomic plant is free of the chloroplast transgene and hence uniformly sensitive to the antibiotic (left). By contrast, emasculated flowers from transplastomic plants pollinated with wild-type pollen give rise to 100% spectinomycin-resistant progeny (right).

with non-transgenic crops; and (b) the possibility of outcrossing through pollen transmission of transgenes from transgenic crop plants to related wild species (for example, from cultivated oilseed rape, *Brassica napus*, to its weedy relative, *Brassica rapa*).[95] Thus, by providing transgene containment, transplastomic technologies ensure much higher ecological safety than classical transgenic technologies involving nuclear genome manipulations.

These advantages of transplastomic plants over conventional transgenic plants make chloroplast transformation technologies a promising tool for biotechnologists which has the potential to solve at least some of the technical problems associated with classical transgenic technologies and, in addition, minimizes the ecological risks upon release of transgenic plants into the environment.[79,97] However, the wide use of transplastomic technologies in plant biotechnology currently encounters one serious drawback: at present, chloroplast transformation is routinely available only for a single higher plant species, tobacco. This is because tobacco is by far the most easy-to-handle species in plant tissue culture, allowing for the development of highly efficient selection and regeneration protocols for the production of transgenic plants. Limitations in the currently available tissue culture systems are considered to be the main obstacle to the extension of transplastomic technologies to other species and, most importantly, to major crop plants. Although recently some progress was made with *Arabidopsis* and potato chloroplast transformation[98,99] as well as with the generation of (heteroplasmic) transplastomic cell lines in rice,[100] a complete protocol for the production of fertile transplastomic plants has not yet been reported for any other species but tobacco. In fact, of the three chloroplast transformants generated to date for the model species of plant geneticists, *Arabidopsis thaliana*, all were sterile and hence could not be propagated generatively.[98] However, with the current acceleration of research in this area, rapid progress with plastid transformation systems for agronomically important plant species will undoubtedly be made in the near future.

For the reason discussed above, all biotechnological research conducted to date with transgenic chloroplasts has been carried out in tobacco. In spite of this limitation, the results of these studies have impressively demonstrated the enormous potential of transplastomic technologies for the biotechnology of the future (reviewed by Hager & Bock;[79] Table 1).

Most crop plants have undergone centuries of breeding. Their efficient cultivation in modern agriculture is largely based on monocultures where plants are exposed to weed competitors as well as viral, bacterial and fungal pathogens. As this results in significant annual harvest losses, the introduction of resistance genes into plant genomes by genetic engineering provides an attractive method of creating highly productive plant varieties not attainable by classical breeding. Taking advantage of the extremely high foreign protein accumulation levels that can be obtained in transgenic chloroplasts, expression of insecticidal proteins[82,84,101] and herbicide-tolerant enzymes[76,83] from the chloroplast genome has proven to be a very efficient strategy for successful resistance management and weed control. For example, insecticidal Bt toxin protein expressed from the tobacco plastid genome accumulated to up to more than 40% of the total soluble cellular protein and the transplastomic plants were highly toxic to insect pathogens in bioassays.[84]

Plants also have considerable potential for the production of pharmaceuticals, edible vaccines and antibodies ("plantibodies"), since they provide a cheap source of protein and various secondary

metabolites (for a review see, e.g. Giddings *et al.*[102]). Recently, the human growth hormone (somatotropin) was successfully expressed from the tobacco plastid genome and shown to accumulated to high levels (>7% of total soluble protein). Interestingly, the eukaryotic protein somatotropin was synthesized in chloroplasts in its correct, disulfide-bonded form and proved to be biologically active in bioassays.[103] This study represents a first promising step towards the use of transplastomic plants as factories for high-yield production of biopharmaceuticals.

For certain applications, it may be desired to restrict plastid transgene expression to a particular tissue or developmental stage. This can be achieved by placing the transgene under the control of a phage T7 RNA polymerase promoter which is normally not recognized by the plastid transcriptional apparatus. Plastid transgene expression can then be switched on by a nuclear-encoded and plastid-targeted T7 RNA polymerase.[104] Expression of the nuclear T7 RNA polymerase gene can, in turn, be controlled by tissue-specific or developmental stage-specific promoters or, alternatively, can be made dependent on chemical inducers of gene expression.[80]

Clearly, transplastomic technologies are still far from being routine tools for plant biotechnologists and some technical limitations have still to be overcome. However, the feasibility studies conducted to date have impressively demonstrated the great potential of plastid genome engineering for a variety of biotechnological applications. High-yield protein and metabolite production as well as effective resistance management are potential areas in biotechnology where transplastomic plants may replace classical transgenic plants in the foreseeable future. Moreover, the increasing need to introduce more than one transgene to express traits determined by multiple genes,[105] will certainly make plastid transformation technologies more and more attractive for plant genetic engineers.

Notes added in proof

A recent report describes the use of a betaine aldehyde dehydrogenase gene as a novel selectable marker for tobacco chloroplast transformation (Daniell, H., Muthukumar, B. & Lee, S. B. (2001). Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. *Curr. Genet.* 39, 109-116).

The Cre/lox site-specific recombination system was successfully used by two laboratories for efficient removal of selectable marker genes from transgenic chloroplast genomes (Hajdukiewicz, P. T. J., Gilbertson, L. & Staub, J. M. (2001). Multiple pathways for Cre/lox-mediated recombination in plastids. *Plant J.* 27, 161-170; Corneille, S., Lutz, K. & Maliga, P. (2001). Efficient elimination of selectable marker genes from the plastid genome by the CRE-lox site-specific recombination system *Plant J.* 27, 171-178).

The most recent development of a plastid transformation system for tomato (Ruf, S., Hermann, M., Berger, I. J., Carrer, H. & Bock, R. (2001). Stable genetic transformation of tomato plastids: expression of a foreign protein in fruits. *Nature Biotechnol.* 19, in press) provides a first system for biotechnological applications of plastid genome engineering in a food crop with an edible fruit.

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Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reduct isomerase and menthofuran synthase

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Peppermint (*Mentha* × *piperita* L.) was independently transformed with a homologous sense version of the 1-deoxy-D-xylulose-5-phosphate reductoisomerase cDNA and with a homologous antisense version of the menthofuran synthase cDNA, both driven by the CaMV 35S promoter. Two groups of transgenic plants were regenerated in the reductoisomerase experiments, one of which remained normal in appearance and development; another was deficient in chlorophyll production and grew slowly. Transgenic plants of normal appearance and growth habit expressed the reductoisomerase transgene strongly and constitutively, as determined by RNA blot analysis and direct enzyme assay, and these plants accumulated substantially more essential oil (about 50% yield increase) without change in monoterpene composition compared with wild-type. Chlorophyll-deficient plants did not afford detectable reductoisomerase mRNA or enzyme activity and yielded less essential oil than did wild-type plants, indicating cosuppression of the reductoisomerase gene. Plants transformed with the antisense version of the menthofuran synthase cDNA were normal in appearance but produced less than half of the undesirable monoterpene oil component than did wild-type mint grown under unstressed or stressed conditions. These experiments demonstrate that essential oil quantity and quality can be regulated by metabolic engineering. Thus, alteration of the committed step of the mevalonate-independent pathway for supply of terpenoid precursors improves flux through the pathway that leads to increased monoterpene production, and antisense manipulation of a selected downstream monoterpene biosynthetic step leads to improved oil composition.

peppermint | *Mentha* × *piperita* | monoterpene biosynthesis | mevalonate-independent pathway | isopentenoids

Isoprenoids are a large and structurally diverse family of compounds that play essential roles in plants as hormones, photosynthetic pigments, electron carriers, and membrane components and that also serve in communication and defense (1). Although isoprenoids are universally synthesized through condensations of the five-carbon compound isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), two distinct and independent biosynthetic routes to these precursors exist in plants. The cytosolic pathway to IPP (Fig. 1*A*) starts from acetyl-CoA and proceeds through the classical intermediate mevalonic acid to provide precursors for the biosynthesis of sesquiterpenes (C_{15}) and triterpenes (C_{30}) (2). The plastidial pathway (Fig. 1*B*) is initiated by the transketolase-type condensation of pyruvate (carbons 2 and 3) and glyceraldehyde-3-phosphate to 1-deoxyxylulose-5-phosphate (DXP), followed by the isomerization and reduction of this intermediate to 2-*C*-methylerythritol-4-phosphate, formation of the cytidine 5′-diphosphate derivative, phosphorylation at C2, and cyclization to 2-*C*-methylerythritol-2,4-cyclodiphosphate as the last defined step (3–6). This plastidial pathway provides precursors for the biosynthesis of isoprene (C_5), monoterpenes (C_{10}), diterpenes (C_{20}), and tetraterpenes (C_{40}) (4, 7), and genes encoding each enzyme of the pathway, up to formation of the cyclic diphosphate, have been isolated from plants and from eubacteria in which the pathway also operates (8–19).

Transgenic manipulations of the mevalonate-independent (DXP) pathway in *Escherichia coli* have indicated that IPP and DMAPP likely arise independently by branching of the pathway (20) and that overexpression of the first pathway gene, for DXP synthase (DXPS), increases carotenoid and ubiquinone biosynthesis (21, 22); manipulation of the mevalonate pathway that operates in yeast also results in increased carotenoid production (23). Studies on the results of overexpression and underexpression of DXPS in *Arabidopsis* have recently indicated that this enzyme catalyzes a slow step in the mevalonate-independent pathway to plastidial isoprenoids (chlorophylls and carotenoids) (24), and considerable literature exists on the transgenic alteration of hydroxymethylglutaryl CoA reductase in plants and the influence on cytosolic isoprenoid production (sesquiterpene phytoalexins and phytosterols); however, the roles of the various reductase isoforms in differentially regulating the mevalonate pathway are not fully clear (25–28). The control of flux through each pathway of isoprenoid biosynthesis in plants, in which both mevalonate and mevalonate-independent (DXP) pathways operate, and the level and means of interaction between the two pathways are of considerable interest in the context of both primary and secondary plant metabolism.

Monoterpenes comprise the major components of the essential oils of the mint family (Lamiaceae), including peppermint (*Mentha* × *piperita*), which has been developed as a model system for the study of monoterpene metabolism. Peppermint oil is chemically complex, and the biosynthetic pathway leading to the major monoterpene component (−)-menthol (Fig. 2) involves a broad range of representative reaction types of terpenoid metabolism (e.g., cyclization, hydroxylation, redox transformations) (29). Monoterpene biosynthesis in mint is specifically localized to the glandular trichomes (30) and originates in the leucoplasts of the secretory cells of these highly specialized nonphotosynthetic epidermal structures (31). During the brief but intense period of secretory activity (32, 33), monoterpene biosynthesis is driven by plastidial supply of IPP and DMAPP via the DXP pathway; the cytosolic mevalonate pathway is also inactive at this stage of oil gland development (34). It is of interest to determine whether flux through the mevalonate-independent pathway is

Abbreviations: DMAPP, dimethylallyl diphosphate; DXP(S), deoxyxylulose phosphate (synthase); DXR, DXP reductoisomerase; IPP, isopentenyl diphosphate; MFS, menthofuran synthase; NPT, neomycin phosphotransferase II; WT, wild type.

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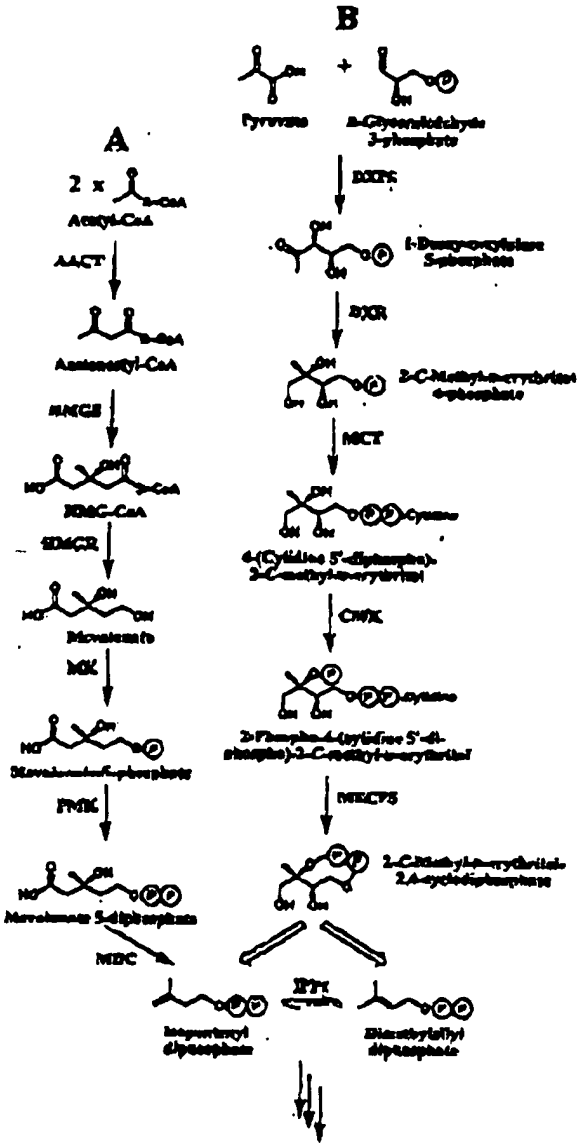

Fig. 1. Biosynthesis of IPP and DMAPP via the *mevalonate* pathway (*A*) and the mevalonate-independent (DXP) pathway (*B*). The indicated enzymes are: AACT, acetyl-CoA/acetyl-CoA C-acetyltransferase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MDC, mevalonate-5-diphosphate decarboxylase; DXPS, 1-deoxyxylulose-5-phosphate synthase; DXR, 1-deoxyxylulose-5-phosphate reductoisomerase; MCT, 2-C-methylerythritol-4-phosphate (MEP) cytidyltransferase; CMK, 4-(cytidine-5′-diphospho)-2-C-methylerythritol kinase; MECPS, 2-C-methylerythritol-2,4-cyclodiphosphate synthase; and IPP isomerase (IPPI). The circled P denotes the phosphate moiety. The large open arrow indicates several as-yet-unidentified steps. The pathway may give rise to IPP and DMAPP independently (20) or the interconversion catalyzed by IPPI.

limiting during the period of very rapid terpenoid biosynthesis by manipulating this route for precursor supply. Such a finding could have important implications for production of the essential oils and other terpenoids of commercial significance (35).

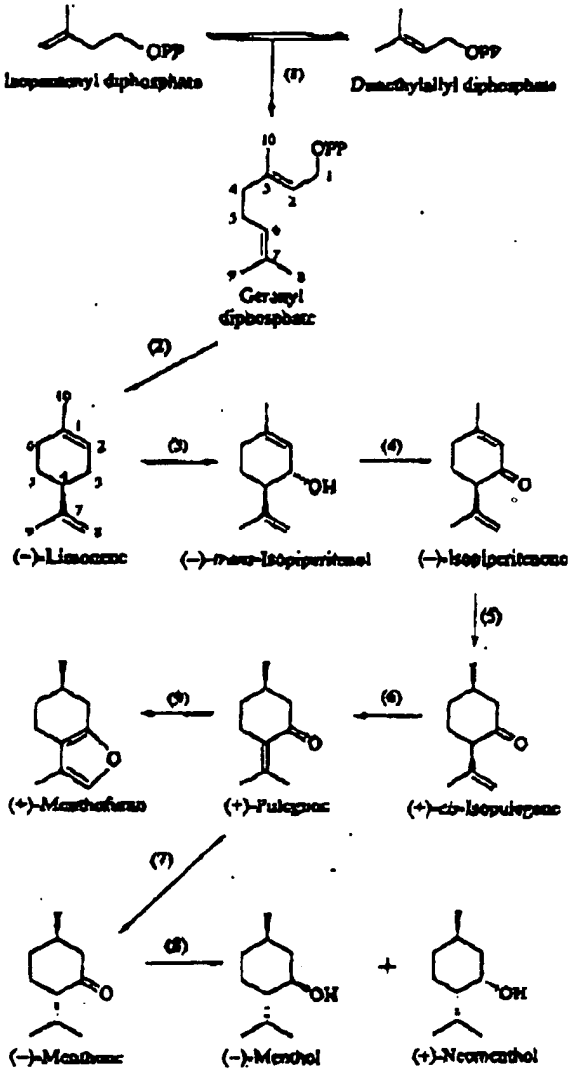

Fig. 2. The principal pathway for monoterpene biosynthesis in peppermint. The responsible enzymes are: 1) geranyl diphosphate synthase, 2) (−)-limonene synthase; 3) cytochrome P450 (−)-limonene-3-hydroxylase; 4) (−)-trans-isopiperitenol dehydrogenase; 5) (−)-isopiperitenone reductase; 6) (+)-cis-isopulegone isomerase; 7) (+)-pulegone reductase; 8) (−)-menthone reductase; and 9) cytochrome P450 (+)-MFS. The circled P denotes the phosphate moiety.

Because DXP is an intermediate not only for IPP and DMAPP biosynthesis but also for the biosynthesis of thiamin and pyridoxol (36, 37), it is the conversion of DXP to methylerythritol phosphate (Fig. 1*B*), catalyzed by DXP reductoisomerase (DXR) (11), that represents the committed step in the production of IPP. In this paper, we report the transformation of peppermint with the homologous cDNA for DXR (12) under the control of a strong constitutive promoter and describe the influence of modified expression of this gene on essential oil production yield and mint physiology.

(+)-Menthofuran is an undesirable monoterpenoid component of peppermint that is derived from the α,β-unsaturated ketone (+)-pulegone (38) (Fig. 2); it contributes off-flavor to the isolated essential oil and promotes off-color on storage (39, 40).

The content of menthofuran can reach industrially unacceptable levels in plants raised under stressful environmental conditions (high temperature, drought, low light intensity) (41, 42), over which commercial mint growers have very limited control. A cDNA-encoding cytochrome P450 (+)-menthofuran synthase (MFS) [(+)-pulegone-9-hydroxylase] was recently isolated from peppermint (38), thus offering a direct, but heretofore unexplored, means for transgenic manipulation of menthofuran production. In this paper, we also report the transformation of peppermint with the antisense version of (+)-MFS (38) under the control of a strong constitutive promoter, and we describe the influence of decreased expression of this gene on the composition of the essential oil produced in stressed and unstressed plants.

Materials and Methods

Plant Material. Peppermint plants (the sterile hybrid *Mentha* × *piperita* L. cv. Black Mitcham) were propagated from rhizomes and stem cuttings in flats containing peat moss/pumice/sand (55:35:10, vol/vol/vol) and were grown under controlled conditions at 500–600 μmol m^{-2} s^{-1} photosynthetically active radiation at plant height, with a 16-h photoperiod and a 26°C/15°C (day/night) temperature cycle (43). To induce moderate stress, which alters oil composition by increasing the levels of (+)-menthofuran and (+)-pulegone (41, 42), the photon flux density was reduced to 200–300 μmol m^{-2} s^{-1}, and the night temperature was increased to 21°C. All plants were watered and fertilized daily with a complete fertilizer (N/P/K, 20:20:20) plus iron chelate and micronutrients, and all flats were grown to complete confluence, then pruned and regrown to maturity (preflowering) before harvesting for oil analysis.

Vector Assembly and Plant Transformation. The parent vector pGAdekG/Nuclear Inclusion-b protein (NIb).L was provided by J. C. Carrington of the Institute of Biological Chemistry. This vector is derived from pGA482 (44) and contains a β-glucuronidase (GUS)-NIb gene fusion inserted between the CaMV tandem 35S promoter with duplicated enhancer and the *Agrobacterium* NOS (transcriptional terminator. The GUS-NIb fusion was excised with *EcoRI*/*KpnI* and replaced by ligation with the DXR cDNA, which was amplified from the original clone (12) by using forward primer (5′-ACTGTCGAATTCATGGCTCTAAACTTGATGGC-3) and reverse primer (5′-ATCGCTGGTACCGCTCATACAAGAGCAGGAC-3) to introduce the respective 5′-*Eco*RI site upstream of the start codon and 3′-*Kpn*I site downstream of the stop codon. The coding region (antisense version) of the MFS cDNA (38) was amplified by PCR by using primers (5-CGCCGCGAATTCTCAAGATTGACGTGGAGTAGC-3) and (5′-CGCCGCGGTACCATGGCCGCTCTTCTAG-3′) to generate an*Eco*RI site and a *Kpn*I site at the respective 3′- and 5′-termini of the gene. The resulting gel-purified amplicon was digested with*Eco*RI and *Kpn*I and ligated into similarly prepared and gel purified pGAdekG/NIb.L to replace the original GUS-NIb insert as before.

The sequence-verified constructs were electroporated into *Agrobacterium tumefaciens* strain EHA105 by using the Micro-Pulser (Bio-Rad) according to the manufacturer's protocol. A single transformant bearing each construct was isolated and grown to log phase in minimal medium (45) containing 50 mg of kanamycin L^{-1} and 30 mg of rifampicin L^{-1}, harvested by centrifugation, resuspended in minimal medium containing 0.2 mM acetosyringone, and used to infect peppermint leaf disks as previously described (46, 47). After regeneration by established protocols (46, 47), rooted plantlets were transferred to soil, acclimated, and then moved to the greenhouse and propagated as above.

RNA Isolation and Blot Analysis. Total RNA was extracted from immature (1–2 cm) and fully expanded (>4 cm) peppermint leaves by using the Trizol Reagent (GIBCO/BRL) according to the supplier's protocol. Ten micrograms of denatured RNA was separated by electrophoresis on a 1.2% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham Pharmacia) by standard protocol (48). ^{32}P-labeled DNA probe, prepared by random priming of the cDNA encoding DXR, was used to detect the corresponding mRNA. Prehybridization was conducted at 65°C for 1 h in 0.5 ml/cm^2 of Rapid Hyb buffer (Amersham Pharmacia), followed by hybridization with the ^{32}P-labeled probe (8×10^6 cpm) under the same conditions for 2 h, and then washing in 4×(15 min, room temperature), 2×(15 min, 65°C), and 1× (15 min, 65°C) SSC containing 0.1% SDS before exposure to Kodak X-Omat x-ray film overnight.

Enzyme Isolation and Assay. Soluble enzyme extracts from peppermint leaves (7–3 cm in length, 0.5 g) were prepared by a standard procedure (49). The resulting soluble enzyme fraction (8 ml) was then suspended with ceramic hydroxyapatite (Bio-Rad, 2 g matrix/8 ml extract) that had been prewashed and equilibrated with extraction buffer [20 mM potassium phosphate (pH 6.5)/10 mM sodium ascorbate/10 mM $MgCl_2$/1 mM DTT]. The slurry was gently mixed for 1 h at 0–4°C toallow protein adsorption, and the matrix was then removed by centrifugation to provide a supernatant essentially free of phosphatase activity that interferes with the DXR assay and neomycin phosphotransferase (NPT) assay. The NPT assay followed an established literature procedure (50). The preparation of the substrate [1-^{14}C]DXP and the details of the radio-HPLC-based assay for DXR activity have been previously described (12).

Essential Oil Analysis. Confluent flats of transgenic mint or wild-type (WT) controls were grown to maturity (flower bud stage) and were individually harvested and frozen at −20°C. The frozen tissue was then manually crushed and mixed to ensure sample uniformity, and three 10-gram samples from each trial (large-stem fragments were excluded) were taken for simultaneous steam distillation–pentane extraction as previously described (43) by using (+)-camphor as an internal standard. One-microliter aliquots of the diluted distillate were analyzed for terpenoid content by gas chromatography (and coupled gas chromatography–mass spectrometry) as described elsewhere (43), and the products were quantified (in milligram/gram tissue fresh weight) by comparison of detector response with that of the internal standard.

Results and Discussion

The first step of the plastidial mevalonate-independent pathway for the production of isoprenoid precursors is catalyzed by DXPS (5, 6), which also supplies precursor (DXP; see Fig. 1) for the synthesis of thiamin and pyridoxol (36, 37). The second step of the pathway is catalyzed by DXR (for the conversion of DXP to methylerythritol phosphate; see Fig. 1), which is considered the committed step in the supply of terpenoid precursors (11) and thus a potential target for control of flux through this branch of the pathway. There have been no previous attempts to manipulate DXR or to evaluate the influence of this or any other gene of the mevalonate-independent pathway on the production yield of essential oil terpenes. A cDNA encoding DXR was isolated from peppermint (12); this 1,425-nt sequence encodes a preprotein bearing an N-terminal plastidial peptide that directs the enzyme to the plastids where the mevalonate-independent pathway operates. The mature enzyme comprises about 400 amino acid residues with a size of about 43.5 kDa, and it resembles other reductoisomerases of plant and eubacterial origin (51).

(+)-MFS was recently demonstrated to be a cytochrome P450 enzyme capable of hydroxylating the*syn* (C9)-methyl group of

(+)-pulegone, which leads to spontaneous intramolecular cyclization to the hemiketal and dehydration to the furan, to yield this commercially undesirable essential oil component (38). An abundant cytochrome P450 clone from a peppermint oil gland cell cDNA library (52) was functionally expressed in *Saccharomyces cerevisiae* and *E. coli* and shown to encode MFS (38), thus offering a transgenic means for control of menthofuran production. The full-length cDNA contains 1,479 nucleotides and encodes a protein of 493-aa residues of molecular weight 55,360, which bears a typical N-terminal membrane insertion sequence and all of the anticipated primary structural elements of a cytochrome P450.

Preparation and Evaluation of Transformed Plants. Genetic transformation of peppermint was accomplished by an established protocol by using *A. tumefaciens* strain EHA105 (46, 47) and a binary vector pGA482 (44) containing *npt* and the full-length (sense) *dxr* construct (12) or the resistance gene and the antisense version of *mfs* (38). Subsequent regeneration and selection from leaf disks transformed with the sense version of *dxr* yielded 57 kanamycin-resistant plants, and of leaf disks transformed with the antisense version of *mfs* yielded 19 kanamycin-resistant plants. Gene transfer in both cases was confirmed directly by assay of leaf extracts for expression of the selectable marker (*npt*) (50), and all NPT-positive plants were propagated for further analysis.

All 19 of the verified transformants bearing the antisense MFS cDNA and most transgenic plants transformed with the DXR (sense) cDNA (42 plants designated the TI group) were indistinguishable from WT plants. In the population of *dxr* transformants, 11 plants (designated the TIIA group) did not develop normal pigmentation; instead, the leaves appeared uniformly lighter green, suggesting that chlorophyll synthesis was impaired. These plants grew more slowly and produced less biomass than did WT. A third group of *dxr*-transformants (four plants designated TIIB) also lacked normal pigmentation in a mosaic pattern.

To determine whether the phenotypic variation observed in the *dxr*-transformants correlated with the expression pattern of the *dxr* transgene, total leaf RNA was isolated for Northern blot analysis by using the DXR cDNA as probe. The results showed that DXR mRNA was strongly expressed in young leaves of WT plants and TI plants (WT appearance) and was easily detected in mosaic plants but not in leaves in which *dxr* was apparently cosuppressed (Fig. 3*A*). In fully expanded leaves, the DXR message was not detectable in WT (or cosuppressed) plants; however, the level of this transcript increased significantly in proportion to total RNA in TI transgenics and was also observed in TIIB mosaic plants (Fig. 3*B*). Because the DXP pathway operates in plastids to supply precursor for the biosynthesis of essential metabolites, such as chlorophyll (4, 6), the high-level expression of *dxr* in young leaves is not surprising. As leaves mature, however, the expression levels of many genes, including *dxr*, might be expected to decrease. In transgenic plants, the DXR cDNA was constitutively expressed under control of the CaMV 35S promoter (53). Thus, as leaves mature and many genes are developmentally silenced, the CaMV 35S promoter remains active, resulting in an increase in the proportion of transgenic DXR mRNA to total leaf RNA and, as a consequence, may maintain DXP pathway function. In the TIIA group, DXR message was not detectably expressed in immature or fully expanded leaves, as determined by Northern blot analysis (Fig. 3), indicating that the *dxr* gene was cosuppressed (54–56) in these plants. Such down-regulation of *dxr* would very likely compromise chlorophyll biosynthesis and result in the phenotypic lack of pigmentation observed.

To assess DXR activity in transgenic plants, DXR assays were performed with soluble protein extracts from developing leaves of plants in each phenotypic category. These results correlated

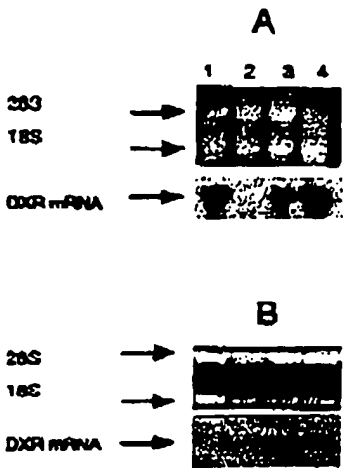

Fig. 3. Measured mRNA levels for DXR in immature (*A*) and fully expanded (*B*) leaves of WT and transgenic peppermint plants. Total leaf RNA was isolated, separated on a denaturing agarose gel (10 μg/lane), blotted, hybridized to the radiolabeled DXR cDNA as probe, and exposed to film (*Lower*). The indicated lanes correspond to: Lane 1, WT plant; Lane 2, transgenic cosuppressed plant; Lane 3, transgenic mosaic plant; and Lane 4, transgenic plant with WT appearance that overexpresses *dxr*. *Upper* illustrates ribosomal bands visualized with ethidium bromide that were used to verify loading of equal amounts of total RNA before transfer.

well with the Northern blot data, in that extracts of TI plants that over-expressed the DXR cDNA contained two to four times more DXR activity (on a nmol$\cdot$h^{-1} mg of protein^{-1} basis) than did the corresponding extracts from WT plants. Conversely, DXR activity was not detected in extracts of plants in which *dxr* was seemingly cosuppressed, although at least low levels of DXR activity must have been present in these plants because they did grow, albeit slowly, and they were not albino.

Effects on Essential Oil Production and Composition. Because glandular trichome metabolism in mint is largely dedicated to monoterpene production driven by precursor supply from the plastidial DXP pathway (7, 12, 34), it was reasoned that alterations in pathway flux because of changes in *dxr* expression should be observable at the level of essential oil accumulation. Essential oil analysis of mint is easily accomplished by steam distillation of leaf tissue followed by gas chromatographic separation of components of the distillate and quantification by using an internal standard (43). These analytical results (Table 1) demonstrated that most plants in the TI group accumulated more oil than WT plants (up to nearly 50% increase in oil yield), whereas plants apparently cosuppressed for *dxr* (TIIA group) produced less oil than did control plants. These analyses further demonstrated that the composition of the essential oil of the transgenic plants was similar to WT in the majority of cases (55 plants). However, two plants produced a significantly different oil composition compared with WT and to the other transgenic plants. One plant (designated DXR16 of the TII group) accumulated higher quantities of menthofuran and pulegone (Fig. 2), whereas a second plant (DXR46 of the TI group) accumulated less pulegone and menthofuran, but more menthol, than did WT plants (Table 1). Additionally, plant DXR46 produced piperitone oxide to a level of about 5% of total oil; this compound was not detected in WT or other transgenic plants. The abnormal oil compositions of DXR16 and DXR46 plants are not consistent with those of other transgenic plants in their respective groups. Thus, it seems likely that these changes in oil profile are not caused by alterations *in dxr* expression but rather are the result of insertional effects of the transgene that

Table 1. Essential oil yield and composition of WT peppermint and selected transformants expressing the sense version of DXP reductoisomerase (DXR plants) and the antisense version of menthofuran synthase (MFS plants)

Plant	Oil yield (mg/g fresh weight)	Percentage					
		Limonene	Cineole	Menthone[a]	Menthofuran	Pulegone	Menthol
WT[b]	1.8	2.4	3.8	45.9	16.8	8.0	6.9
DXR6	2.6	2.0	3.4	45.0	13.7	6.1	12.7
DXR7	2.3	2.0	3.8	55.8	7.2	3.2	11.0
DXR8	2.4	1.9	3.9	45.0	15.5	5.7	12.6
DXR16	1.4	1.8	3.0	23.6	36.0	16.8	8.5
DXR32	2.6	2.7	3.9	46.1	12.5	5.7	13.0
DXR37	2.7	2.2	3.8	47.9	14.7	7.3	13.3
DXR38	2.0	2.0	4.6	50.7	11.7	5.3	13.2
DXR40	2.7	1.7	3.1	62.8	13.6	5.2	13.3
DXR44	2.4	1.9	3.3	38.6	15.0	6.7	11.0
DXR46	1.7	4.8	4.8	45.3	5.1	1.7	27.0
WT[c]	2.3	1.9	4.6	64.0	3.0	2.0	8.5
MFS1	1.7	1.1	5.3	35.0	2.5	0.2	23.1
MFS3	1.4	1.7	5.8	63.7	2.5	0.7	12.7
MFS7	2.4	1.3	6.3	53.5	2.5	0.8	19.5
MFS15	1.8	1.8	4.0	65.2	3.2	1.1	10.0
WT[d]	1.7	2.3	4.2	60.2	13.9	7.8	4.0
MFS7	1.8	2.6	5.0	68.8	5.3	2.8	7.3

All measurements represent the averages of three replicates of two independent tissue samples, SE ≤ 10%. Each group of transformants was compared to WT plants grown under the same conditions.

[a]Isomenthone is not included. The combination of menthone plus isomenthone generally constitutes 60–70% of the oil.

[b]This oil composition is typical of newly established plants raised under these moderate stress growth conditions.

[c]This oil composition is typical of newly established plants raised under these unstressed growth conditions.

[d]This oil composition is typical of established plants raised under these moderate stress growth conditions.

serve, directly or indirectly, to down-regulate pulegone reductase (DXR16) and MFS (DXR46) (see Fig. 2).

In the case of peppermint plants transformed with the antisense version of *mfs*, most (15 plants) produced an oil of near average composition and yield compared with WT (data not shown). However, four of these plants (MFS1, 3, 7, and 15) accumulated 35–55% less (+)-menthofuran (and 40–60% less (+)-pulegone), and substantially more (−)-menthol, than WT controls (Table 1). Oil evaluation over a period of 6 months (four independent distillations and analyses) demonstrated that the MFS7 transgenic plant consistently produced an oil of comparable yield with lower levels of menthofuran and pulegone, and higher levels of menthol, than WT plants. This pattern of uncompromised oil yield and compositional modification persisted even when plants were grown under stress conditions (obtained by elevated night temperature combined with decreased photon flux during the daylight period) that are known to promote the production and accumulation of menthofuran and pulegone (41, 42) (Table 1). It is notable that peppermint plants transformed with *mfs* in antisense orientation (MFS1, 3, 7, and 15) produce an essential oil very similar in composition to the DXR46 plant transformed with the sense version of the reductoisomerase (Table 1), suggesting that the latter bears an insertion that inactivates the *mfs* gene to produce a similar oil compositional change.

Conclusions

The present results directed to the manipulation of *dxr* as the committed step of the mevalonate-independent pathway to terpenoids support previous findings (24, 57, 58) with *Arabidopsis* in which disruption of *dxps* (the *cla1* gene encoding the first step of the mevalonate independent pathway) led to early arrest of chloroplast development and an albino phenotype. In the present instance, both essential oil and chlorophyll biosynthesis were impaired in the *dxr* cosuppressed plants, but it was clear from the visible phenotype and essential oil chemotype that precursor supply from the DXP pathway was not entirely eliminated in these plants.

Transgenic up-regulation of *dxr*, as evidenced by Northern blot analyses and direct DXR enzyme assays, led to an increase in essential oil accumulation, a result that may be attributed to improved flux of precursors for monoterpene biosynthesis in the oil glands by the increased level or developmental duration of the DXP pathway. Either effect implies that DXR catalyzes a slow step of the mevalonate-independent pathway. It is notable that essential oil yield increases approaching 50% did not result in observable changes in the complex oil composition noted for most plants. This coupling of yield increase without compositional change indicates that the capacity for limonene production (and downstream biosynthetic steps; see Fig. 2) has not been exceeded and thereby suggests that additional rate-determining step(s) reside somewhere between DXR and limonene synthase (the first committed step of monoterpene biosynthesis).

Transgenic down-regulation of *mfs*, by the antisense approach, led to the anticipated decrease in oil content of (+)-menthofuran (without change in yield) but surprisingly did not increase (+)-pulegone content as might be expected via the decreased conversion of this ketone intermediate to (+)-menthofuran (see Fig. 2). Rather, a decrease in the oil content of *both* menthofuran and pulegone was observed in the transgenic antisense MFS plants (Table 1). This unusual observation is currently unexplained but nevertheless represents a favorable compositional change, because both menthofuran and pulegone are considered undesirable monoterpene components when present in peppermint essential oil at levels exceeding a few percent.

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ORIGINAL PAPER

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Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase

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Abstract The recently discovered non-mevalonate pathway to isoprenoids, which uses glycolytic intermediates, has been modulated by overexpression of *Escherichia coli* D-1-deoxyxylulose 5-phosphate synthase (DXS) to increase deoxyxylulose 5-phosphate and, consequently, increase the isoprenoid precursor pool in *E. coli*. Carotenoids are a large class of biologically important compounds synthesized from isoprenoid precursors and of interest for metabolic engineering. However, carotenoids are not ordinarily present in *E. coli*. Co-overexpression of *E. coli dxs* with *Erwinia uredovora* gene clusters encoding carotenoid biosynthetic enzymes led to an increased accumulation of the carotenoids lycopene or zeaxanthin over controls not expressing DXS. Thus, rate-controlling enzymes encoded by the carotenogenic gene clusters are responsive to an increase in isoprenoid precursor pools. Levels of accumulated carotenoids were increased up to 10.8 times the levels of controls not overexpressing DXS. Lycopene accumulated to a level as high as 1333 μg/g dw and zeaxanthin accumulated to a level as high as 592 μg/g dw, when pigments were extracted from colonies. Zeaxanthin-producing colonies grew about twice as fast as lycopene-producing colonies throughout a time course of 11 days. Metabolic engineering of carbon flow from simple glucose metabolites to representatives of the largest class of natural products was demonstrated in this model system.

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Introduction

Isoprenoids are a diverse group of natural products found in all organisms. They are derived from the C_5 skeleton of isopentenyl pyrophosphate (IPP). IPP was thought to be strictly derived from the mevalonate pathway, and the enzyme HMGCoA reductase was considered to be a rate-limiting enzyme for this biosynthetic route (Dimster-Denk et al. 1994; Chappell 1995). Recently, a novel non-mevalonate biosynthetic pathway was discovered that operates specifically in plastids of plants (reviewed in Lichtenthaler 1999) as well as in bacteria and in cyanobacteria; in plants, the mevalonate pathway operates in the cytoplasm in parallel with the plastid-localized non-mevalonate IPP pathway (Rohmer et al. 1993; Schwender et al. 1996). In the non-mevalonate route, IPP is derived from deoxyxylulose 5-phosphate (DXP), which in *Escherichia coli* has also been found to be a common precursor in the biosynthesis of vitamins B_1 (thiamin) and B_6 (pyridoxal) (Sprenger et al. 1997). The enzyme D-1-deoxyxylulose 5-phosphate synthase (DXS) is responsible for catalyzing the synthesis of DXP from pyruvate and GAP (glyceraldehyde 3-phosphate) (Sprenger et al. 1997; Lange et al. 1998; Lois et al. 1998). In plants and bacteria, competition for DXP might affect end-product accumulation for different isoprenoid-derived biosynthetic pathways. The enzyme responsible for DXP synthesis might represent a novel rate-controlling enzyme whose expression could be modified to increase substrate availability for pathways under consideration for metabolic engineering.

Carotenoids are one example of isoprenoids for which interest in metabolic engineering relates to their usefulness as coloring agents and as precursors to vitamin A and to retinoids, compounds essential to growth and development (Misawa et al. 1991, 1993; Yamano et al. 1994; Burkhardt et al. 1997; Ruther et al. 1997; Wang et al. 1999). Carotenoids are synthesized in certain bacteria, fungi, and in plastids of plants (as reviewed in Armstrong and Hearst 1996; Cunningham and Gantt

1998). Gene clusters encoding the carotenoid biosynthetic enzymes have been isolated from epiphytic bacteria such as *Erwinia uredovora*, and their introduction into *Escherichia coli* has resulted in caroten id accumulation (Misawa et al. 1990). In addition to the prospect of bioengineering the accumulation of useful and unusual carotenoids, the expression of carotenogenic genes in *E. coli* represents a unique opportunity for the cloning of new genes by heterologous complementation (Sun et al. 1996), for the functional testing of gene products (Li et al. 1996) and for the examination of flow through the pathway (Kajiwara et al. 1997; Ruther et al. 1997).

The C_{20} isoprenoid geranylgeranyl pyrophosphate (GGPP), produced by GGPP synthase (GGPPS), is the first precursor to the carotenoids, and to a variety of other isoprenoid-derived products, including gibberellins, the phytol chain of chlorophyll, prenylquinones, tocopherols, prenylated proteins, and many secondary metabolites such as taxol and casbene (Chappell 1995). GGPP is formed from four units of IPP, one of which is an isomer (DMAPP). IPP isomerase converts IPP to DMAPP in *E. coli*; DMAPP condensed with consecutive m lecules of IPP forms farnesyl pyrophosphate (FPP), geranyl pyrophosphate (GPP) and then GGPP.

In *E. coli*, the presence of GGPP depends on the activity of endogenous IPP isomerase and FPP synthase, and exogenous GGPPS. Recent efforts have focused on increasing the carotenoid accumulation by overexpression of these three enzymes (Wang et al. 1999), as well as by overexpression of enzymes within the carotenoid pathway (Kajiwara et al. 1997; Ruther et al. 1997). Hypothetically, the accumulation of carotenoids also depends on the concentration of IPP as derived from the non-mevalonate pathway. Since the DXS enzyme functions at the junction of three pathways, manipulation of DXS enzyme levels might be as important in enhancing end-product accumulation as in increasing levels of

gene cluster present in plasmids pACCRT-*EIB* or pACCAR-25Δ*crtX*, alternatively named pCAR25*delX* (Misawa et al. 1990), as previously described (Wurtzel et al. 1997). Plasmid pACCAR25Δ*crtB* (Misawa et al. 1990), which has a frameshift mutation in the gene encoding phytoene synthase, produces no carotenoid and was used as a negative control. These carotenoid-accumulating strains were transformed using the $CaCl_2$ protocol (Sambrook et al. 1989) with an additional plasmid, pTAC-ORF2 (*dxs*) expressing *E. coli* D-1-deoxyxylulose-5-phosphate synthase (Lois et al. 1998). Bacteria were cultivated in Luria-Bertani (LB) medium in liquid culture or agar plates (Sambrook et al. 1989) supplemented with 50 μg/ml ampicillin to select for pUC-derived plasmids carrying *dxs* and 70 μg/ml chloramphenicol to select for pACY184-derived plasmids carrying the *Erwinia* carotenogenic gene clusters. In some cases DXS expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG). Liquid cultures were inoculated from a glycerol stock (Sambrook et al. 1989) prepared from one primary transformant colony. A 10-μl aliquot of the glycerol stock was added to 10 ml LB with antibiotic and grown to stationary phase for 12 h at 37 °C with shaking at 240 rpm. Aliquots (50 μl) of these starter cultures were then inoculated into 50 ml LB with antibiotics in 125-ml Erlenmeyer flasks, incubated at 37 °C with shaking for 30 h, then held at room temperature for an additional 30 h. For growth of colonies on solid medium, cells from a glycerol stock of approx. 500 primary transformant colonies scraped from a plate and mixed together were serially diluted and plated out to LB plates with antibiotics at a density of approx. 150 colonies per 15 × 100-mm Petri dish. These plates were incubated at 37 °C for 8 h and then at room temperature for 2–11 days. All cultures were grown in the dark and shielded from light during all manipulations and subsequent carotenoid extractions.

Carotenoid analysis

Colonies lifted on to dry 5.4-cm^2 nitrocellulose membranes or single colonies lifted onto 7-mm-diameter discs were placed colony-side up in a pool of 50 mM glucose/0.25 mM Tris (pH 8.0) 10 mM EDTA (GTE) on a glass plate or microtiter plate lid and scanned wet on a Hewlett Packard ScanJet 6100C at the following settings: contrast, 200; brightness, 200; scaling, 300%; resolution, 100 dpi; 256 greys. Colony boundaries were selected manually and pixel values determined with ImageQuaNT V4.1b (Molecular Dynamics, Sunnyvale, Calif.). After scanning, cells were released from the

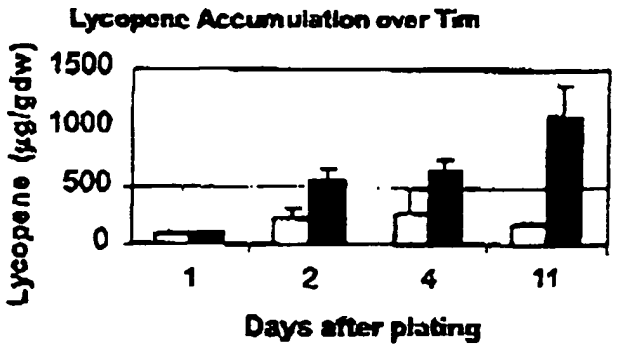

Fig. 2 Time course of carotenoid accumulation in strains containing pACCRT-*EIB* (□) or pACCRT-*EIB* + pTAC-ORF2(DXS) (■). Values are averages of 2–4 determinations and error bars are ±SD

The differences observed when strains were grown on solid medium were further explored by means of growth in liquid culture. Extracts from liquid cultures showed a range of pigment expression similar to that measured in colonies, but with much greater variation (data not shown). We saw a large variation in carefully prepared replicate cultures, as well as large variation among cultures prepared from different primary transformants. IPTG induction of DXS expression at various time points had a negative effect on carotenoid accumulation in liquid cultures as well as in colonies on plates (data not shown).

Discussion

Accumulation of both lycopene and zeaxanthin in *E. coli* was greatly enhanced by overexpression of DXS, suggesting that IPP pools may be limited by DXS expression and activity. Other studies have shown effects on the rate of carotenoid synthesis by causes downstream of the IPP pool. For example, Wang et al. (1999) have shown that co-overexpression of heterologous GGPPS (CrtE) and homologous IPP isomerase, enzymes upstream of the carotenoid biosynthetic pathway but downstream from DXS, increased astaxanthin accumulation in *E. coli* up to a level of 234 µg/g dw. Co-overexpression of genes encoding a novel bifunctional GGPPS (*gps*) and IPP isomerase (*idi*) led to a drastic increase in astaxanthin levels up to 1419 µg/g dw, demonstrating that GGPPS and IPP isomerase are rate-controlling. By modulating IPP precursor levels in the presence of native levels of IPP isomerase and FPP synthase, we obtained a maximum value for accumulation of lycopene that was similar (1333 µg/g dw). Together, these studies clearly indicate that flux into the heterologous carotenoid biosynthetic pathway is influenced by rate-controlling enzyme levels which are also responsive to precursor concentrations.

Overexpression of enzymes within the carotenoid biosynthetic pathway has not led to large increases of carotenoid accumulation. For example, co-transformation of carotenogenic strains harboring pACYC-derived plasmids with an additional pUC-derived plasmid overexpressing one of enzymes of the biosynthetic gene cluster (such as phytoene synthase, phytoene desaturase or lycopene cyclase) had a negative effect on carotenoid accumulation. Only carefully controlled (by growth-phase-specific IPTG induction) overexpression of a terminal enzyme, β-carotene hydroxylase, created a metabolic sink and a 1.3-fold increase in zeaxanthin accumulation to 276 µg/g dw (Ruther et al. 1997).

In contrast, we report a 2.2- to 10.8-fold increase, relative to our average values of extracted carotenoid, when DXS is overexpressed. While our average values for accumulation in Δ*crtX* and *EIB* are similar to values for other carotenoid gene cluster-containing *E. coli* strains in the literature (Ruther et al. 1997; Wurtzel et al. 1997; Wang et al. 1999), we measured increases up to 592 µg/g dw for zeaxanthin and up to 1333 µg/g dw for lycopene with overexpression of DXS. pTAC-TAC expression vectors are IPTG-inducible, but lack tight control of the basal expression level from the strong P_{tac} promoter, leading to DXS expression in the absence of induction (Rohmer et al. 1993). Preliminary experiments with induction of DXS expression by inclusion of IPTG in solid medium or addition of IPTG to liquid cultures at inoculation, at mid-log phase and at stationary phases of growth had a negative effect on lycopene- and zeaxanthin accumulation.

Inclusion of glucose in the growth medium increases astaxanthin pigmentation in *E. coli* overexpressing GGPPS and IPP isomerase in combination with an astaxanthin biosynthetic gene cluster (Wang et al. 1999). Co-overexpression of IPP isomerase in our DXS-overexpressing lines in the presence of glucose may further stimulate carotenoid accumulation in our strains.

Success with manipulation of these coupled pathways in bacteria may be applicable to plants, especially as the carotenoid biosynthetic pathway and the non-mevalonate route to IPP via DXS are plastid-localized. Efforts are underway to extend metabolic engineering of the isoprenoid precursor pool in the presence of a carotenoid biosynthetic sink to the higher plants.

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The biotechnological potential and design of novel carotenoids by gene combination in *Escherichia coli*

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Carotenoids are antioxidants with considerable pharmaceutical potential. More than 600 carotenoid structures are known but their availability is limited owing to practical difficulties associated with chemical synthesis and isolation from microorganisms or plant tissue. To overcome some of these problems, heterologous expression of carotenoid genes in *Escherichia coli* can be used for the synthesis of rare derivatives or even of novel carotenoids. Novel and rare carotenoids can be obtained by combining carotenoid genes from different host species in *E. coli*.

Carotenoids are naturally occurring pigments synthesized as hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) by plants and microorganisms. Their major function is in protection against oxidative damage by quenching photosensitizers, interacting with singlet oxygen[1] and scavenging peroxy radicals[2], thus preventing the accumulation of harmful oxygen species.

This protective process is dependent on their chemical structures, which can differ in the length of the polyene chromophore, the nature of the end groups and the various substituents that they contain[3]. The exact chemical structure of individual carotenoids is decisive for their biological properties because it determines how they interact with other molecules and integrate into membranes[4].

The role of carotenoids

Over the past 20 years, evidence has accumulated that carotenoids play an important role in the prevention of cardiovascular disease and cancer[5–7]. For example, skin-cancer-induced mice and rats that were fed or injected with β carotene and canthaxanthin showed a significant delay in tumor growth. In other animal studies, protection against other types of cancer (e.g. of the salivary gland, colon or mammary gland) was observed. More than 600 naturally occurring carotenoids have been identified to date but most of these are biosynthetic intermediates that accumulate only in trace amounts, making it very difficult to extract sufficient material for their purification and application in animal studies. The number of carotenoids available for evaluation of their pharmaceutical potential is thus very limited.

In addition to animal studies, several cell-assay systems for the chemoprevention of cancer have been developed[8]. These assays are convenient models to study protective effects on morphological transformation, communication and the interaction of cancer cells. Such systems are also extremely useful in evaluating relatively small amounts of carotenoids. At present, the main limitation to these studies is the supply of structurally diverse carotenoids. It is thus important to search for more-effective carotenoids and to carry out studies on the relationship between structure and activity.

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Other uses of carotenoids

Industrial applications of carotenoids include their use as nutrient supplements, for pharmaceutical purposes, as food colorants and in animal feeds. The actual sale of carotenoids is estimated to be approximately US$500 million and the market is increasing[9]. To date, the majority of carotenoids used industrially are chemically synthesized (e.g. astaxanthin, canthaxanthin and β carotene), although some are also available as natural extracts. In addition, β carotene and astaxanthin are also produced commercially by microbial fermentation but their market share is not very significant[10]. However, as more complex carotenoids reveal their pharmaceutical potential, fermentation processes using natural or genetically modified microorganisms will be indispensable in their production.

Recent developments in the molecular biology of carotenoid biosynthesis from organisms that accumulate different carotenoid products have provided a variety of genes[11] that can be employed as tools for a new strategy of heterologous expression in different host organisms. A combination of genes from organisms that follow different branches of a pathway makes it possible to synthesize novel compounds. This strategy only works, however, when the substrate specificity of the enzyme is such that it does not need to recognize the entire substrate molecule but only certain regions of it that are suitable for conversion. A detailed biochemical characterization of the substrate and product specificity of expressed enzymes[12] is necessary to choose the appropriate genes. This approach to the production of new compounds by gene combination in a heterologous host has proved to be successful for the synthesis of carotenoids and also for tetracenomycines and related antibiotics, which are formed via the polyketide pathway[13].

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Escherichia coli as a factory for carotenoids

Recently, carotenoids have been successfully synthesized in non-carotenogenic bacteria and yeast using recombinant gene techniques[14–17]. Considerable progress has been made, especially in the expression of all the necessary genes to synthesize structurally different carotenoids such as lycopene, β carotene and zeaxanthin in *E. coli*[14,18].

The potential of this system is determined by several factors. For high-yield carotenoid production, optimization should focus on several different aspects. First, sufficient precursors (i.e. substrates for the reactions involved) should be available. Second, a balanced level of carotenogenic enzymes should be expressed, to enable efficient precursor conversion without the formation of intermediate metabolite pools. Third, the correct plasmid combination is important to minimize the accumulation of intermediates and to increase the yield of the end product (e.g. in zeaxanthin formation[19]). Last, the host organism should exhibit an active central terpenoid pathway and possess a high storage capacity for carotenoids.

Available genes and plasmids

Carotenogenic genes or cDNAs are available from bacteria, algae, fungi and higher plants, most of which can be functionally expressed in *E. coli* (Table 1). Owing to the small number of genes that have so far been cloned from the pathway leading to carotenoids with 30 carbon atoms[20], only diapophytoene and diaponeurosporene can be produced. However, for the synthesis of carotenoids with 40 carbon atoms, the choice of genes is much broader.

In all cases, *crtE* and *crtB* from *Erwinia* species are used in *E. coli* to form phytoene, which is the first carotenoid in the C_{40} pathway. A variety of phytoene-desaturase genes are available in order to achieve different degrees of desaturation of the acyclic molecule (Table 1). Different genes for lycopene cyclase, which is responsible for the formation of β and ε rings, have been cloned and other genes have already been used to insert hydroxy and keto groups into cyclic and acyclic carotenes. To synthesize a particular carotenoid, the necessary genes can either be combined in a single plasmid or inserted into individual plasmids. The only known carotenogenic gene that cannot be expressed in *E. coli* is the zeaxanthin epoxidase from *Nicotiana*[21], this is not functional in *E. coli* because this prokaryotic host cannot provide reduced ferredoxin, which is needed for epoxide formation[22].

One advantage to using *E. coli* for carotenoid production is its capacity for transformation with several plasmids as long as they all possess a different origin of replication (i.e. the plasmids have to belong to different incompatibility groups). Furthermore, it is essential that each plasmid carries a different antibiotic resistance marker and that selection pressure be maintained at a high level to prevent spontaneous plasmid loss. Useful vectors belonging to different incompatibility groups for constructing expression plasmids for carotenogenic genes include pBR322-related plasmids like pUC (pMB1 origin of replication, ampicillin resistance[23]), pACYC184 (p15A origin of replication, chloramphenicol resistance[24]), pRK404 (RK2 origin of replication, tetracycline resistance[25]) and pBBR1MCS2 (SC101 origin of replication, kanamycin resistance[26]).

Metabolic optimization of terpenoid-precursor production

Carotenoids are formed via the terpenoid pathway; in *E. coli*, the only known terpenoid products are the prenyl side chains of ubiquinone and menaquinone, and the dolichols, which are involved in bacterial-cell-wall synthesis[27]. Carotenoid biosynthesis mediated by

Table 1. The genes and enzymes available for the synthesis of different carotenoids and their precursors in *Escherichia coli*

Enzyme	Substrate	Reaction product	Refs
C_{30} chain			
Diapophytoene synthase	FPP	Diapophytoene	20
Diapophytoene desaturase	Diapophytoene	Diaponeurosporene	20
C_{40} chain			
GGPP synthase	FPP	GGPP	12
Phytoene synthase	GGPP	Phytoene	30
Desaturases	Phytoene	ζ Carotene	36
	Phytoene	Neurosporene	36
	Phytoene	Lycopene	36
	Phytoene	3,4-Didehydrolycopene	12
	ζ Carotene	Lycopene	34,37
	Hydroxyneurosporene	Demethylspheroidene	38
Lycopene cyclase	Lycopene	β Carotene	18,39
	Lycopene	ε Carotene	40
Hydroxylase	β Carotene	Zeaxanthin	18
Ketolases	Spheroidene	Spheroidenone	38
	β Carotene	Canthaxanthin	41
	β Carotene	Echinenone	42
Hydratase	Neurosporene	1-Hydroxyneurosporene	38
Glycosylase	Zeaxanthin	Zeaxanthin diglucoside	18

Abbreviations: FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

foreign genes in *E. coli* must compete with these endogenous pathways for terpenoid precursors. This diversion of early prenyl pyrophosphates from endogenous pathways into carotenoids can be alleviated by genetic engineering of the metabolic pathway in *E. coli*.

It was recently shown that, in *E. coli*, the isomerization of isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate is limiting in the terpenoid pathway. The gene for IPP isomerase has been cloned and found to enhance the synthesis of carotenoids when expressed in *E. coli* with a carotenoid background[28]. This implies that, in *E. coli*, isomerization of IPP to dimethylallyl pyrophosphate, which subsequently serves as a substrate for chain elongation, is a major regulatory step in the formation of different terpenoids. Consequently, the amounts of carotenoids synthesized are dependent on the activity of IPP isomerase expressed in *E. coli*.

In addition to the removal of this metabolic bottleneck, there is also competition for geranylgeranyl pyrophosphate (GGPP), which is the substrate for phytoene synthase. Induction of high-level expression of the *crtB* gene for an enzymatically active phytoene synthase in *E. coli* decreased the growth of the expressing strain by draining off GGPP from the endogenous metabolic processes[29] because the capacity for GGPP synthesis is very low in *E. coli*[29]. This problem could be overcome by cotransformation with a GGPP-synthase gene[18].

It has been shown that high-level expression of the GGPP-synthase gene enhanced the synthesis of carotenoids by about 20% compared with low-level expression[17], demonstrating that provision of GGPP is a second limiting step for the synthesis of carotenoids. When both GGPP-synthase and IPP-isomerase levels were increased by simultaneous introduction of the corresponding genes[28], enough GGPP and other precursors are provided for the synthesis of essential housekeeping terpenoid compounds in addition to carotenoids in *E. coli*.

Examples of gene combinations and the resulting carotenoids

It has recently been demonstrated that it is possible to synthesize eight different hydroxy derivatives of carotenoids in *E. coli*[28], including the acyclic carotenoids 1-hydroxyneurosporene, 1-hydroxylycopene, 1,1'-dihydroxylycopene and dimethylsperoidene, and the cyclic carotenoids 3-hydroxy-β-zeacarotene, 7,8-dihydrozeaxanthin, 3- or 3'-hydroxy-7,8-dihydro-β-carotene and 1'-hydroxy-γ-carotene. Most of these carotenoids are found only in trace amounts in natural sources. For the synthesis of all the carotenoids mentioned above, *E. coli* was transformed with a combination of up to three compatible plasmids, which contained several carotenogenic genes from *Erwinia uredovora* and two *Rhodobacter* species. The structures of these carotenoids and the pathway of their formation is outlined in Fig. 1. Finally, all new or uncommon carotenoids should be rigorously characterized by different spectroscopic methods such as mass and NMR spectroscopy[29].

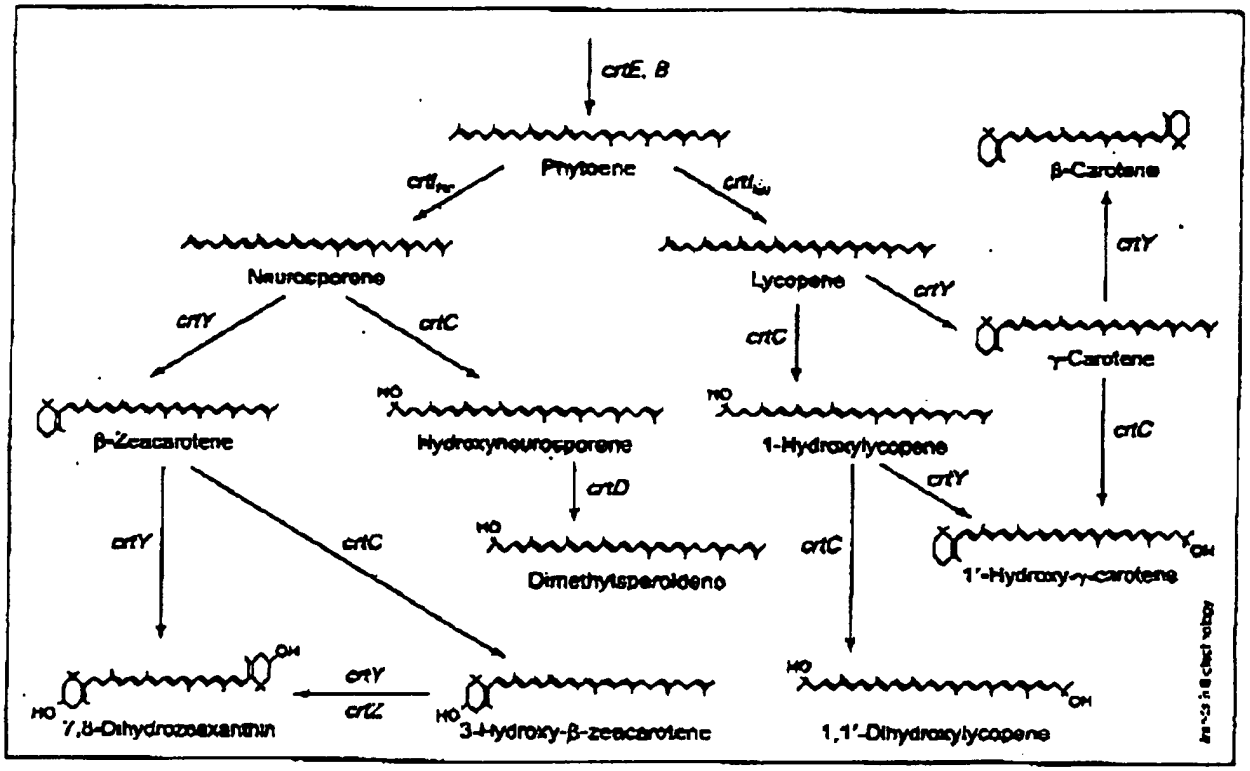

Figure 1

Structures and route of formation of hydroxy carotenoids in *Escherichia coli* transformants, that carry genes from the bacteria *Erwinia* and *Rhodobacter*. Genes encoding the enzymes that catalyse individual reactions are indicated next to the arrows.

Conditions for carotenoid production

Carotenoid formation is dependent on the strain and the culture medium; the media of choice were those with up to 2.5% casein hydrolysate as the major carbon source. Growth conditions must be optimized in order to produce different hydroxy carotenoids in amounts that justify their isolation and purification[31]; yields of hydroxy carotenoids obtained were around 100 μg (g dry weight)$^{-1}$. Generally, carotenoid concentrations were higher at lower temperature. This inverse relationship of carotenoid formation to the growth temperature of *E. coli* can be attributed to slower expression of carotenogenic genes, yielding more-active enzymes and also to a better provision of prenyl pyrophosphates as carotenoid precursors when the entire metabolism is slower. Good carotenoid yields were obtained during a growth period of 48 h at 28°C.

The growth and formation of lycopene (an example carotenoid) is shown in Fig. 2. A typical logarithmic growth curve is evident, with stationary phase being attained after approximately 45 h at 28°C. The increase of carotenoid synthesis per cell is slower and proceeds in a linear fashion up to 40 h, resulting in a yield of approximately 0.5 mg (g dry weight *E. coli*)$^{-1}$. Also, the incorporation of an additional isopentenyl-pyrophosphate-isomerase gene from the unicellular green alga *Haematococcus pluvialis*[32] results in an additional 1.5–2 times increase in carotenoid synthesis.

In some cases, intermediates may form up to half of the carotenoid end product[31]. In these instances, rigorous separation and purification is necessary, leading to further losses of the desired end product, although, in many cases, the intermediates obtained during purification may prove to be as valuable as the desired carotenoid when evaluated in pharmaceutical tests.

Extraction and purification of carotenoids

The large-scale extraction and purification of carotenoids from *E. coli* pose a number of specific problems that have to be resolved[20]. For example, lipophilic impurities from the bacterial host have to be separated from the carotenoids. On a laboratory scale, lyophylized bacterial cells can be ground in a cell mill and extracted with acetone for 8–12 h in the dark to avoid carotenoid degradation. After removal of the acetone, the residue is dissolved in a diethylether–petrol mixture or, in case of hydroxycarotenoids, in acetone–petrol and purified by passing through a silica-gel column. Suitable solvent mixtures for elution are diethylether–petrol (1:4 v/v) for carotenes and acetone–petrol (1:4 v/v) for hydroxy-carotenoids. To determine carotenoid purity in isolated fractions, HPLC is the most-appropriate technique[26].

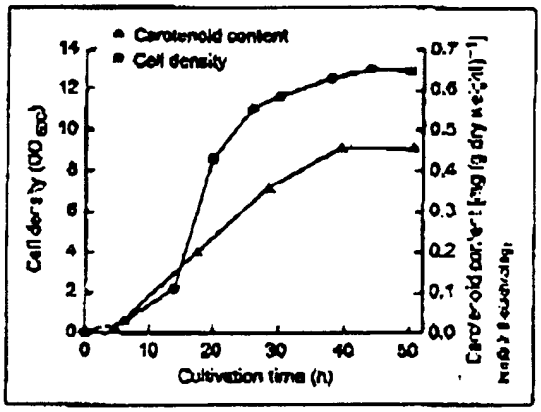

Figure 2

Production of the carotenoid lycopene by recombinant *Escherichia coli* JM101 transformed with pACCRT-EB$_{Pa}$ at 28°C in TB medium containing 1.7% casein hydrolysate, 0.8% yeast extract, 0.4% glycerol, 0.23% KH_2PO_4 and 1.25% K_2HPO_4. Abbreviation: OD_{600}, optical density at 600 nm.

Future outlook

The combination of carotenogenic genes results in a diversity of carotenoid structures that cannot be synthesized by conventional chemical methods. Presently, only a small number of carotenoids can be obtained as pure compounds in significant amounts in order to study their biological function in *in vivo* and *in vitro* assay systems. An evaluation of their antioxidant properties, including protection against singlet oxygen or radicals, as well as their potential as anticancer agents will follow. For their large-scale production, further research is necessary, which will also require a more-detailed understanding of the growth parameters in fermenters. One of the major problems is the extraction and purification process. However, optimized gene constructs will limit the accumulation of carotenoid intermediates, which complicate the purification process and decrease the yield of the desired carotenoids.

Carotenogenic genes are available from various organisms, mostly bacteria. The construction of plasmids with appropriate promoters and the transformation of *E. coli* strains are complex tasks but are not likely to be a major problem. Accordingly, in the near future, many vectors may be constructed for new and hitherto-unknown carotenoids with different chain lengths, multiple hydroxy and keto groups, aromatic rings, glycosides, and/or esters. At present, the early steps in the terpenoid metabolism of *E. coli* are not well understood[33] but, once the pathway from 1-deoxy-D-xylulose to isopentenyl pyrophosphate is elucidated, this knowledge can be used to increase the precursor supply for carotenoid synthesis by genetic modification of the host.

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Engineering baker's yeast: room for improvement

Francisca Randez-Gil, Pascual Sanz and Jose A. Prieto

Bread making is one of the oldest food-manufacturing processes. However, it is only in the past few years that recombinant-DNA technology has led to dramatic changes in formulation, ingredients or processing conditions. New strains of baker's yeast that produce CO_2 more rapidly, are more resistant to stress or produce proteins or metabolites that can modify bread flavour, dough rheology or shelf-life are now emerging.

Saccharomyces cerevisiae strains have been selected for many years for their dough-leavening characteristics. Baker's yeast produces the CO_2 that results in dough leavening and contributes to the flavour and crumb structure of bread. The yeast gassing rate is critical in baking technology, and this depends on the dough formulation, on specific fermentation parameters and especially on intrinsic characteristics of baker's yeast.

However, there is a general lack of knowledge of the genes responsible for the specific properties of baker's yeast[1,2]. Most or all of these traits appear not to be under the control of single genes or even a limited number of genes. In addition, industrial baker's yeasts are homothalic, with a high and irregular degree of polyploidy and a low level of sporulation. Consequently, the improvement of strains that play a role in fermentation is still one of the most important biotechnological challenges in baker's yeast.

Frozen-bread-dough and, in particular, frozen-sweet-dough production have increased notably during the past few years. The storage of frozen bread dough has a negative influence on baking performance, increasing the proofing time and reducing the bread volume[3]. These effects are mostly a consequence of the loss of viability and reduced gas-production capacity of the yeast cells that arises from freeze injury[4,5]. Consequently, it is common for bakers to add extra yeast to frozen dough, which increases the cost of the process. Therefore, specific strains of baker's yeast that are

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Genetic manipulation of carotenoid biosynthesis: strategies, problems and achievements

Gerhard Sandmann

Carotenoids, some of which are provitamin A, have a range of diverse biological functions and actions, especially in relation to human health. For example, carotenoids are known to be crucial for normal vision and have been associated with reducing the risk of several degenerative diseases including cancer. The putative advantage of modifying and engineering the carotenoid biosynthetic pathways is obvious: to provide sources for the isolation of desired carotenoids or to generate food plants with increased carotenoid content. This article reviews the studies of carotenoid production in heterologous microorganisms and the engineering of crop plants using manipulated carotenoid biosynthesis.

Carotenoids comprise a group of naturally occurring pigments that attract the eye by making flowers and fruit colorful. Structurally these pigments are terpenoids derived by condensation of prenyl pyrophosphates. Carotenoids are lipophilic compounds and can be stored in a lipophilic environment. They are synthesized and sequestered in plastids. The typical C_{40} carotenoids carry cyclic β-ionone end groups that can be substituted by oxo, hydroxy and epoxy groups at different positions (Fig. 1). The most significant part in the molecule is the conjugated double bond system that determines their color and is responsible for their biological action. Their prominent function is as antioxidants owing to their potential to inactivate singlet oxygen and to quench carboxy radicals. Evidence is accumulating that carotenoids play an important role in human health by preventing degenerative diseases. Carotenoids with unsubstituted β-ionone end groups are precursors of vitamin A. The industrial use of carotenoids involves their application in nutrient supplementation, for pharmaceutical purposes, as food colorants and in animal feeds. A recent review covers various aspects of carotenoid properties, as well as their dietary role, potential in disease prevention and biosynthesis.

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Carotenoid demand and supply

The commercial demand of carotenoids is mainly met by chemical synthesis and to a minor extent by extraction from natural sources. However, the supply is restricted to a few carotenoids. Some important dietary carotenoids are not abundant in our food and cannot be taken as supplements. Zeaxanthin, for example, is a rare carotenoid, which, together with lutein, is the essential component of the macular pigment in the eye. Low levels of carotenoid intake increases the risk of age-related macular degeneration.

During ingestion of carotenoids, the efficiency of absorption is strongly dependent on the type of food, the way the food is processed and the amount of accompanying dietary lipids. When carotenoids are added as a food supplement, the problem of intestinal absorption can be overcome by an appropriate formulation. Whether the presence of a carotenoid in the food matrix might facilitate its bioavailability is still not known.

The enormous progress in the cloning of carotenogenic genes opens up the possibility of modifying and engineering the carotenoid biosynthetic pathways in microorganisms and in plants. Within the past year, the potential for carotenoid production in heterologous microorganisms has been shown using several different molecular biology approaches, as well as the engineering of crop plants with manipulated carotenoid biosynthesis. However, a successful manipulation of a pathway for higher metabolite levels or novel compounds faces three basic problems:

- Precursors are consumed at the expense of existing pathways.
- Interference with a well balanced regulation pathway might occur.
- Product storage, especially when highly lipophilic compounds such as carotenoids are generated, must be ensured.

Therefore, high-yield carotenoid production should focus on increased precursor supply, maintaining the balance of interacting metabolic pathways and the use of organisms or targeting of tissues that are capable of incorporating lipophilic molecules.

When nutritional supplementation of a rare carotenoid is the primary goal, two general strategies can be followed. One possibility is fermentative production, such as in a modified microorganism followed by product isolation and addition to processed food. Alternatively, the desired carotenoid can be synthesized in food plants and consumed directly.

Transgenic microorganisms

Carotenoids have been successfully synthesized in the non-carotenogenic yeast *Candida utilis* which has systematically been genetically modified as a production host for lycopene, β-carotene and astaxanthin[1]. The foreign bacterial carotenoid biosynthesis genes were altered according to the codon usage of *C. utilis* and expressed under the control of constitutive promoters derived from the host. Carotenoid formation could be further increased by metabolic engineering of the early mevalonate pathway to increase the supply of terpenoid precursors and also by decreasing the use of prenyl

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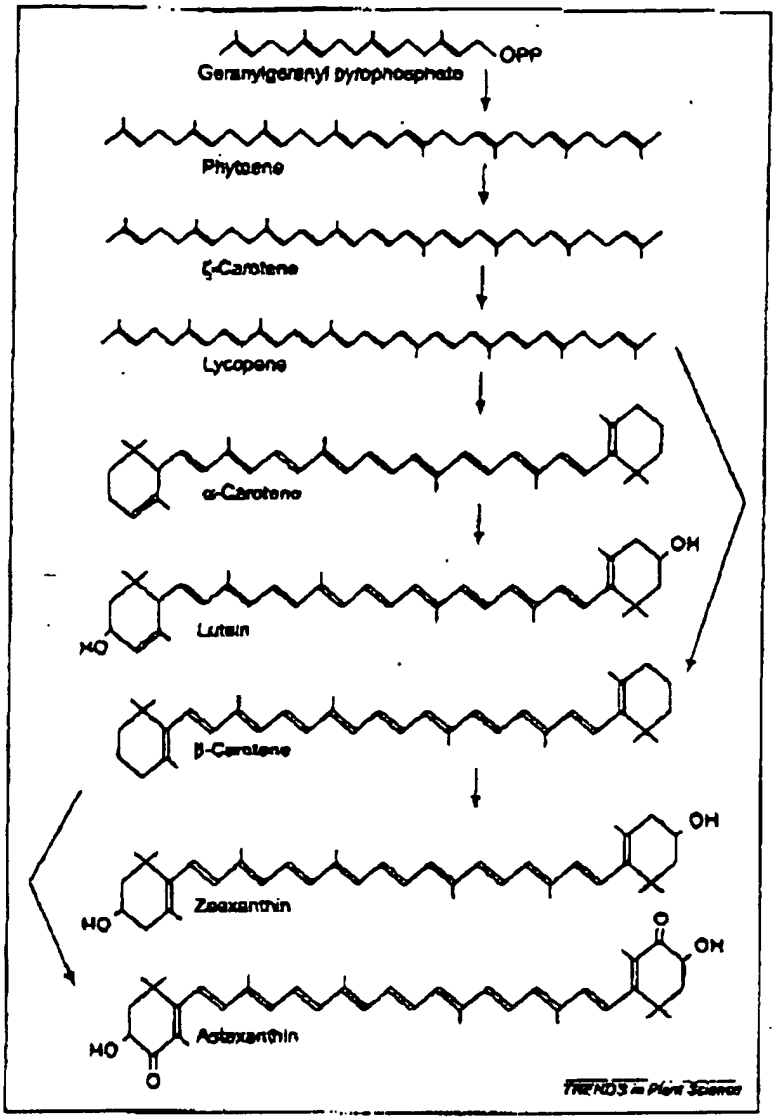

Fig. 1. Biosynthetic pathway of α- and β-carotene and their oxo derivatives. Formation of β-carotene is universal in bacteria, fungi and plants. It initially involves a condensation reaction followed by four desaturation steps and the cyclization of both ends (β-ionone rings). α-Carotene carries only one β- and an additional ε-ionone ring. Other carotenoids of biotechnological interest are derived from α- and β-carotene by hydroxylation at positions 3 and 3′ (lutein and zeaxanthin) and/or formation of positions 4 and 4′ such as astaxanthin.

pyrophosphates through the competitive sterol pathway[3]. This engineered strain yielded 7.8 mg per g dry weight of lycopene.

E. coli is a convenient host for heterologous carotenoid production. Most of the carotenogenic genes from bacteria, fungi and higher plants can be functionally expressed in this bacterium. Furthermore, plasmids belonging to different incompatibility groups with different antibiotic resistance markers are available. They can all be introduced simultaneously in *E. coli* for carotenoid synthesis, enabling combinations of individual genes to be used. The potential of *E. coli* as a carotenoid production system has been reviewed recently. The problem of precursor supply can be overcome by the metabolic engineering of its deoxyxylulose 5-phosphate pathway, which provides the prenyl pyrophosphates for carotenoid synthesis. Transformation with the genes for over-expression of 1-deoxy-D-xylulose 5-phosphate synthase, 1-deoxyxylulose 5-phosphate reductoisomerase and isopentenyl pyrophosphate isomerase, stimulated carotenogenesis by up to 3.5-fold to a final yield of 1.5 mg per g dry weight[6]. Similar concentrations were reached for astaxanthin in an *E. coli* strain that had been modified in a different way[8]. Increasing precursor supply seems to be lethal, indicating that the limitation of carotenoid storage has been reached.

A combination of genes from different organisms that follow different branches of the pathway makes it possible to synthesize novel compounds. However, this strategy only works when the substrate specificity of the enzyme is such that it does not need to recognize the entire substrate molecule but only certain regions of the molecule that are suitable for conversion. This combinatorial approach can result in the formation of numerous novel carotenoids including 1-hydroxylated acyclic structures with up to 13 conjugated double-bonds[7]. Their antioxidative potential is superior to other related carotenoids.

Another way of acquiring genes for the synthesis of novel carotenoids is to modify them towards a modified substrate and/or product specificity using molecular breeding. Phytoene desaturase genes from two *Erwinia* species were shuffled randomly and a recombined gene obtained encoding an enzyme that introduced six instead of four double-bonds[9]. In addition, a lycopene cyclase was obtained with several amino acid exchanges that can convert 3,4-didehydrolycopene into the corresponding monocyclic product torulene.

Transgenic plants

Different approaches have been followed to modify the carotenoid content in plants to enhance the nutritional value:

- Modification of the carotenoid pathway by shifting to another carotenoid product in tomato[4].
- Increasing the amounts of pre-existing carotenoids in rape (*Brassica napus*) seed[10].
- Engineering a carotenogenic pathway in tissue that is completely devoid of carotenoids such as rice endosperm[11].

Synthesis of phytoene by condensation of two molecules of geranylgeranyl pyrophosphate is the initial step in carotenoid biosynthesis (Fig. 1). During lycopene deposition in tomato fruit ripening, the activity of phytoene synthase is the major controlling factor of the pathway. Therefore, this enzyme was a prominent target for the genetic manipulation of the carotenoid composition of tomato fruit[2]. The constitutive high level expression of phytoene synthase in tomato resulted in carotenoid-rich seed coats, cotyledons and hypocotyls. However, plants

were reduced in stature because of changes in the gibberellic and abscisic acid contents. In this case, the amounts of gibberellins decreased because of competition for prenylpyrophosphates by both pathways. This work illustrates how problems arise when a balanced metabolism is disturbed.

Manipulation of the desaturation activity in tomato was achieved with a bacterial phytoene ζ-carotene desaturase gene[9]. Its expression lowered the total amount of carotenoids in transgenic tomato fruit to some extent but resulted in a threefold increase in the β-carotene content. Other carotenogenic enzymes, especially the endogenous ζ-carotene desaturase and the lycopene β-cyclase, were upregulated by elevated transcript levels.

Other unexpected results were obtained upon transformation of tobacco with an alga β-carotene ketolase gene[3]. The construct involved the promoter of a phytoene desaturase gene from tomato, which is a single gene in the genome, thus contributing to carotenoid synthesis in fruit and in leaves. Nevertheless, formation of ketocarotenoids was absent in the leaves of the transformant, which might be because of a comparable low expression activity of the promoter. Expression was high in flowers as visualized by the red nectary pigmentation caused by astaxanthin and other ketocarotenoids. Total carotenoid levels were increased to 140% compared with the wild type.

Rice grains are devoid of carotenoids. Genetic modification leading to the formation of cyclic carotenoids (e.g. β-carotene) in the rice endosperm should establish at least four different reactions catalyzed by the enzymes phytoene synthase, phytoene desaturase, ζ-carotene desaturase and lycopene cyclase (Fig. 1). Transformation was carried out with a plasmid containing a plant phytoene synthase gene and a bacterial phytoene desaturase gene, which together should mediate the synthesis of lycopene from geranylgeranyl pyrophosphate[11]. Both reading frames were extended with transit sequences for the targeting of the endosperm plastids. One was under the control of the endosperm-specific glutelin the other under the constitutive cauliflower mosaic virus 35S promoter. Surprisingly, the accumulated carotenoids were lutein, zeaxanthin and β-carotene instead of lycopene. Obviously, the lycopene β- and ε-cyclases and the β- and ε-hydroxylases must have been induced in the transformant or are expressed constitutively. Co-transformation with a lycopene β-cyclase containing plasmid increased the β-carotene content of the rice endosperm to a maximum level of 1.6 μg per g dry weight.

The genetic manipulation of rape seeds to increase the carotenoid content to high levels was a tremendous success. Overexpression of a bacterial phytoene synthase gene extended with a plastid-targeting sequence under a seed-specific promoter increased the carotenoid content of mature rape seed by up to 50-fold[10]. In the transformant the embryos were bright-orange compared with the green embryos in the control. In the transgenic seeds, concentrations of carotenoids (mainly α- and β-carotene) of more than 1 mg per g fresh weight accumulated (the value on a dry weight basis will be several fold higher), yielding an oil with 2 mg per g carotenoids.

Conclusion and prospects

Genetic manipulations of carotenoid biosynthetic pathways have been applied to microbial carotenoid production systems and to crop plants to increase and modify carotenoid accumulation.

Production of carotenoids in suitable microorganisms is versatile. Combinations of new genes and those modified by *in vitro* genetic recombination will lead to a wealth of different carotenoid derivatives for structure–function investigations. Metabolic engineering can avoid bottle necks in the supply of terpenoid precursors. Furthermore, the regulatory circuits directing the metabolic flux to an engineered carotenoid pathway can be redesigned[4]. The major problem for reaching high yields is the storage capacity of the hosts. Engineering carotenoid-sequestering systems into bacteria and fungi still lies ahead of us. When this is resolved, fermentative production of carotenoids in heterologous hosts should lead to a commercial success.

Several attempts have been made to genetically manipulate carotenoid biosynthesis in crop plants, thus increasing the nutritional value, but care still has to be taken not to disturb other isoprenoid pathways. This can be avoided by targeting tissue in which the primary metabolism is rather low, such as fruit and seeds, with organ-specific promoters. Although the biochemistry of carotenogenesis has been well established over the past decades, unexpected new details such as involvement of a plastidic terminal oxidase in the desaturation of carotenoids and in chlororespiration have been discovered recently (reviewed on pp. 31–36 of this issue). There might be more unknown connections of carotenogenesis to other metabolic pathways, which should be revealed and considered in future engineering of carotenoid biosynthesis.

The major obstacle to precisely modifying carotenoid metabolism by introducing *trans* gene is our limited knowledge of how expression of endogenous carotenogenic genes is regulated in higher plants. We are still in the learning phase of understanding how and why endogenous carotenogenic genes are expressed upon the introduction of foreign genes.

Several authors have referred to vitamin A deficiency as a stimulus for engineering a higher β-carotene content in food plants. Looking at the concentrations that were reached in some transgenic plants, the levels are low in comparison to other β-carotene-containing plant tissue such as leaves and carrot roots (up to 6 mg per g dry weight β-carotene, together with ~3 mg per g dry weight α-carotene)[1].

In the case of tomato, the β-carotene concentrations in tomato varieties with high levels of β-carotene obtained by conventional breeding are definitely higher[15] than in the transgenic fruit described above. However, the tremendous increase in β-carotene concentration in modified rape seeds yielding a β-carotene-enriched oil is a great achievement[9]. This is an encouraging milestone on route to manipulating carotenoid biosynthesis and other metabolic pathways in plants.

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